PRION SPECIES BARRIER AT THE SHORT PHYLOGENETIC DISTANCES IN THE YEAST MODEL

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PRION SPECIES BARRIER AT THE SHORT PHYLOGENETIC DISTANCES IN THE YEAST MODEL

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DEDICATION

I dedicate this thesis to my parents, Mr. Guoqing Chen and Mrs. Wei Zhao. This work would not exist without your constant love, faith, support and encouragement.

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LIST OF SYMBOLS OR ABBREVIATIONS

5-FOA 5-fluoroorotic acid
α-aa α-aminoadipic acid
aa amino acid
ade1-14$_{SC}$ *ade1-14* of *S. cerevisiae*
AE aggregation element
ATCC The American Type Culture Collection
Amp ampicillin
BSE bovine spongiform encephalopathy
Ca_2Cl calcium chloride
Cm chloramphenical
$CuSO_4$ copper sulfate
DTT dithiotreitol
EDTA ethylenediaminetetraacetic acid
EtBr ethidium bromide
FAME fundamental and applied molecular evolution
Gal galactose
GFP green fluorescent protein
Glu glucose
GuHCl guanidine hydrochloride
IPTG isopropyl-β-thiogalactopyranoside
KCl potassium chloride
LB luria broth
LiCl lithium chloride
$MgCl_2$ magnesium chloride
MnCl manganese chloride
MOPS morpholinopropanesulfonic acid
NaCl sodium chloride
ORF open reading frame
ORs oligopeptide repeats
PE propagation element
PMSF phenylmethylsulfonyl fluoride
Prion proteinaceous infectious particles
PrP prion protein
QN QN-rich stretch
Raf raffinose
RbCl rubidium chloride
RFP red fluorescent protein
SDS-PAGE SDS-polyacrylamide
SDS sodium dodecyl sulfate

SUMMARY

Prions are self-perpetuating and, in most cases, aggregation-prone protein isoforms that transmit neurodegenerative diseases in mammals and control heritable traits in yeast. Prion conversion requires a very high level of identity of the interacting protein sequences. Decreased transmission of the prion state between divergent proteins is termed “species barrier” and was thought to occur due to the inability of divergent prion proteins to co-aggregate. Species barrier can be overcome in cross-species infections, for example from “mad cows” to humans. We studied the counterparts of yeast prion protein Sup35, originated from three different species of the *Saccharomyces sensu stricto* group and exhibiting the range of prion domain divergence that overlaps with the range of divergence observed among distant mammalian species.

Heterologous Sup35 proteins co-aggregated in the *S. cerevisiae* cells. However, *in vivo* cross-species prion conversion was decreased and *in vitro* polymerization was cross-inhibited in at least some heterologous combinations, thus demonstrating the existence of prion species barrier. Moreover, the barrier between the *S. cerevisiae* protein and its *S. paradoxus* and *S. bayanus* counterparts was asymmetric both *in vivo* and *in vitro*. Our data show that a decreased cross-species prion transmission does not necessarily correlate with a lack of cross-species co-aggregation, suggesting that species-specificity of prion transmission is controlled at the level of conformational transition rather than co-aggregation.

Sup35 could be divided into three domains, and one of them, called prion domain, is sufficient for the species barrier among the *S. sensu stricto* species. We constructed *SUP35* chimeric prion domains, combining the subregions of various origins, and showed chimeric Sup35p retains the capability of forming prions, and in different cross-species combinations, different modules of prion domain play a crucial role in the controlling of species-specificity of prion transmission. One essential amino acid position has been identified by further investigation using site-directed mutagenesis in *S. cerevisiae* and *S. paradoxus* system. Our data support a model suggesting that identity of the short amyloidogenic sequences is crucial for the species barrier.

Sup35 originated from three different species of the *S. sensu stricto* group were capable of forming a prion in *S. cerevisiae*. However, it was not known whether or not they are capable of generating and maintaining the prion state in the homologous cell environment. We have constructed the *S. paradoxus* and *S. bayanus* strains with the reporter allele *ade1-14* (UGA) of *S. cerevisiae* (*ade1-14*$_{SC}$), enabling us to detect [*PSI*$^+$]. We have also fused the N-proximal fragments of *S. paradoxus* or *S. bayanus* Sup35 to a highly hydrophobic human membrane protein, which have been proven to induce respective intact Sup35 proteins into a prion state in the *S. cerevisiae* cells in the absence of another prion, [*PIN*$^+$], which is usually required for *de novo* [*PSI*$^+$] induction but is not present in *S. paradoxus* and *S. bayanus*. By using this system, we were able to demonstrate *de novo* [*PSI*$^+$] formation in *S. paradoxus* but not in *S. bayanus*. Our data show that [*PSI*$^+$] formation is not a unique property of *S.*

cerevisiae, and demonstrate the role of species-specific cellular factors in control of prion properties.

CHAPTER 1

Introduction: Background and Significance

1.1 Prions are infectious proteins

Prion (proteinaceous infectious particles) is a protein isoform that is able to convert the normal form of the same protein into a prion form, which is thought to undergo a self-perpetuating change from a soluble form to a highly ordered fibrous β-rich aggregated, amyloid-like, form (Figure 1.1). Prions are implicated in infectious neurodegenerative diseases, such as "mad cow disease", or bovine spongiform encephalopathy (BSE), sheep scrapie disease, and human Creutzfeldt-Jacob disease. The abnormal prion protein (PrP) is the sole component that is responsible for the genesis and transmission of a disease (5, 6). The mammalian PrP typically contains five remarkably conserved octapeptide repeats (7). The properties of PrP are very similar to those seen in various non-infectious amyloidoses resulting from conversion of certain proteins or their fragments from the normally soluble form to insoluble fibrils or plaques, which places prion diseases into the large and heterogeneous group of amyloid diseases, including near 20 human diseases, such as Alzheimer, Huntington and Parkinson diseases (8, 9).

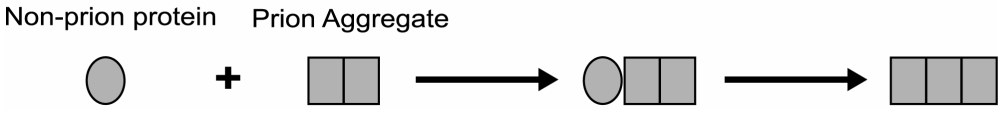


Figure 1.1 Prion model
Prion aggregate (rectangle) is able to convert a non-prion protein (ellipse) into a prion.

1.2 "Species barrier" prevents or decreases cross-species prion transmission

Studies of the mammalian prions have highlighted the importance of sequence specificity in amyloid propagation. A species barrier prevents PrP^{SC} (from scrapie), a prion isoform of normal protein PrP^{C} (cellular), derived from one species from infecting another, for example, the transmission of scrapie from sheep to human, and hamster prion to mouse (10, 11) (Figure 1.2 A-B). However, the species barrier is not absolute and could be overcome. BSE agent appears to be able to cross the barrier and infect human, which demonstrates the importance of studying the cross-species prion transmission (12) (Figure 1.2 A). The species barrier has been studied in mammalian system both *in vivo* and *in vitro*. In nature, mouse prions infect hamster, but not *vice versa* (10) (Figure 1.2 B). The *in vitro* assay for the "seeded" propagation of PrP amyloid has been applied to the study of species barrier. Bu using purified PrP^{*} (derivative of PrP containing amino acid residues 23-144), it was found out that hamster PrP^{*} amyloids seed hamster and mouse PrP^{*} polymerization, but not human PrP^{*} polymerization, while mouse and human PrP^{*} amyloids work on mouse and human PrP^{*}, but not on hamster PrP^{*} (Figure 1.2 C). Mouse PrP^{*} amyloids seeded by the hamster PrP^{*} protein are capable of seeding hamster but not human PrP^{*} polymerization (13, 14) (Figure 1.2 D). The results are impressive, however, some questions remain to be answered, such as: 1) why the *in vitro* cross-seeding barrier between mouse and hamster works in the opposite direction compared to the *in vivo* data; 2) why the species barrier exhibits both asymmetric and symmetric patterns; and so on.

1.3 Yeast prions control heritable traits

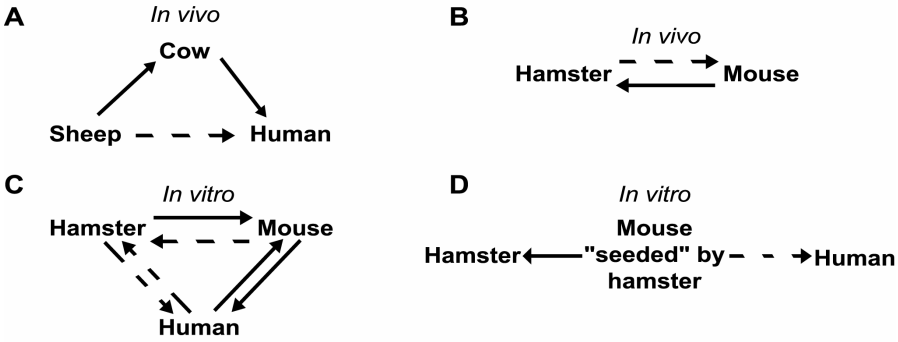


Figure 1.2 Prion species barrier *in vitro* and *in vivo*
Arrows indicate transmission of prion (amyloid) properties, and dashes indicate lack of transmission.

Prion capability is not restricted to PrP and can be detected in the other proteins. Yeast prion proteins control inheritance of non-Mendelian traits transmitted via cytoplasmic infection. Yeast prion [PSI^+], an aggregated form of Sup35 protein (also called eRF3), is one of the most extensively studied yeast prions, and the normal form of this protein is a translation termination factor. Another well-studied yeast prion [URE3] is the aggregated form of the normal protein Ure2 which regulates nitrogen catabolism. More yeast and fungal prions were identified, and one of them, [PIN^+] (or called [RNQ^+]), is the prion isoform of normal protein Rnq1 with an unknown function (15, 16). [PIN^+] promotes [PSI^+] formation in the strains overproducing Sup35 or Sup35N (prion domain, see below, Figure 1.3) (17-20). [NU^+], another example of yeast prions, is supported by a chimeric protein that has the first 123 amino acid of Sup35 replaced with the first 153 amino acid of New1 (21). [Het^S], a prion isoform of Het-s, has been identified in fungus *Podospora* to control cytoplasmic incompatibility, and is the first example of prion switch controls a normal biological important function (22).

1.4 Sup35 structure and function

Sup35 could be divided into three domains. N-proximal prion domain (Sup35N, N) is required for prion induction and propagation; middle domain (Sup35M, M) has an unknown function; and C-proximal domain (Sup35C, C) is essential for translational termination and viability (18, 23, 24) (Figure 1.3 A).

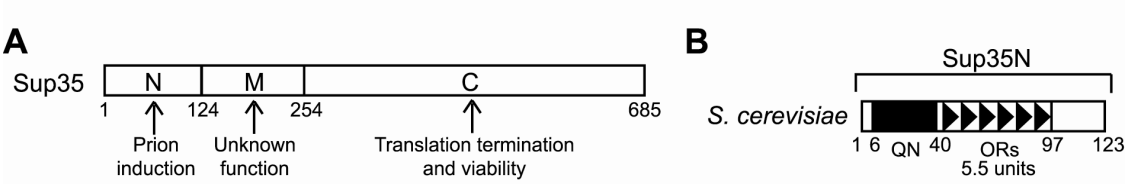


Figure 1.3 Structural and functional organization of the Sup35 protein
N, M and C refer to Sup35N, Sup35M and Sup35C regions, respectively. Numbers correspond to amino acid positions. (A) Structural and functional organization of the Sup35 protein. (B) Structure of the Sup35N region (prion domain) from *S. cerevisiae*. QN and ORs refer to the QN-rich stretch and oligopeptide repeats, respectively.

Sup35N could be subdivided into QN-rich stretch (QN); oligopeptide repeats (ORs) and the last about 25 amino acids without apparent sequence pattern (Figure 1.3 B). QN manifests itself as an aggregation element (AE), which resembles poly-Q proteins and promotes polymerization via intermolecular interactions, while ORs region constitutes a propagation element (PE) that resembles PrP and is essential for the propagation of an amyloid state in cell generation (15, 25). Comparative study of the *SUP35* genes from 10 more distantly related budding yeast species revealed that higher degree of variability among Sup35NM sequence, but confirmed that QN and ORs regions remain most conserved, and C region exhibits high degree of homology (26).

Because the [*PSI*$^+$] state partially inactivates the translational termination factor, Sup35, the most convenient phenotypic assay for [*PSI*$^+$] is translational readthrough, or nonsense suppression. There are some nonsense alleles, and one of the most used alleles, *ade1-14* (UGA) of *S. cerevisiae* (*ade1-14*$_{SC}$), can be suppressed by [*PSI*$^+$] to such an extent that growth on the medium lacking adenine (-Ade) is seen in a reasonable period of time. In [*psi*$^-$] cells, the absence of functional Ade1 protein prevents growth on –Ade and causes the accumulation of a red pigment on rich medium YPD (yeast extract, peptone and dextrose), which in [*PSI*$^+$] cells, readthrough of *ade1-14*$_{SC}$ leads to growth on –Ade and pink for "weak" variant or white color for "strong" variant on YPD (17) (Figure 1.4).

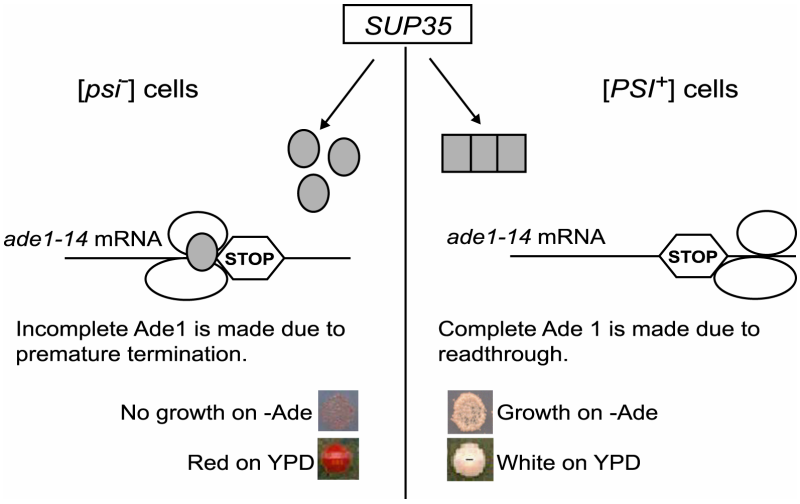


Figure 1.4 Experimental assay used to monitor presence of the yeast prion [*PSI*$^+$] In a [*psi*$^-$] cell, Sup35 remains soluble and therefore readily available to participate in activities related to translation termination. In a [*PSI*$^+$] cell, Sup35 is sequestered into an aggregated form, increasing readthrough of nonsense codons by translating ribosomes. In contrast to [*psi*$^-$] cells, [*PSI*$^+$] cells are able to generate significant quantities of the Ade1 product from the *ade1-14*$_{SC}$ transcript. Absence of the Ade1 product is indicated in [*psi*$^-$] cells as absence of growth on –Ade medium and red color on YPD, and accumulation of Ade1 is manifested in [*PSI*$^+$] cells as growth on –Ade medium and a white color on YPD.

Strong [*PSI*$^{+}$] variants are white on YPD medium and grow fast (in 2-3 days) on –Ade, while weak variants are medium-pink on YPD and grow slowly (in 4-7 days) on –Ade. Strong variant is usually 100% stable in mitotic divisions, whereas weak variant produces some [*psi*$^{-}$] colonies in nonselective conditions (18).

1.5 Models of prion fiber structure

Currently, there are three structural models of yeast and fungal prion fibers (27). First one is based on the structure of a fibrillar microcrystals formed by peptide, GNNQQNY, from Sup35 residues 7-13. The peptide is arranged in a double layer of β-sheets. Each sheet is held together by a network of hydrogen bonds between side chains and between the backbones. In addition, a new interaction named a "steric zipper" holds the two sheets together. This is proposed to be a basic feature of amyloid-like fibrils and helps explain their amazing stability (28) (Figure 1.5).

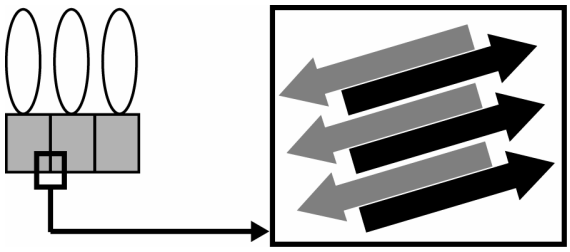


Figure 1.5 Structural organization of amyloid fibers
Functional regions (white circles) are exposed on the side and could be properly folded. Prion domains (gray squares) form an axis. The monomers are held together by the interdigitations of side chains. The strands (gray and black arrows) in each sheet (gray and black) are parallel and precisely aligned, and the two sheets are antiparallel relative to one another.

The second model is suggested by the structure of the aggregating region of Het-s, which is composed of two homologous pairs of β-strands, each forming a β-strand-turn-β-strand motif. Startlingly, the four β-strands within one protein molecule can be arranged in two layers of two-stranded β-sheets (29).

The last model is based on the structural studies of fibers formed by Sup35NM fragment. A core sequence composed of head, central and tail regions is cooperatively folded and protected from solvent. The only intermolecular interactions are head-to-head and tail-to-tail. The molecule then enters the amyloid state and fibers grow by head-to-head and tail-to-tail additions (30).

1.6 Species barrier in *Saccharomyces/Pichia, Saccharomyces/Candida* and Sup35/New1 systems

The prion phenomenon [*PSI*$^+$] offers a powerful system to study the mechanism of species specific prion transmission. The prion domain is quite conserved, and the prion forming capability is retained in yeast evolution. *SUP35* from *Pichia methanolica* (distantly related to *S. cerevisiae*) is able to acquire the [*PSI*$^+$] state in *S. cerevisiae*, but can not transmit it to *S. cerevisiae* Sup35 protein, which is indicative of a species barrier between *Saccharomyces* and *Pichia* (31). The species barrier is also detected, both *in vivo* and *in vitro,* between *Saccharomyces* and *Candida*. The species specificity was found to be encoded in a short region of the prion domain located at QN region, and the prion

species barrier correlated with the inability of heterologous prion proteins to coaggregate (21, 32-34).

A chimeric prion domain composed with the aggregation-determining QN-rich traits (NYN repeat) of New1 and ORs of Sup35 showed that NYN repeat was sufficient for specific interaction, and the mismatched sequences outside of the aggregating region did not prevent cross-interactions between heterologous proteins (15, 25, 35). This agrees with the role of Sup35 QN in controlling species barrier.

1.7 Prion divergence within the genera *Saccharomyces* mimics the mammalian system

We have developed a new experimental system for studying prion species barrier that employs the closely related Sup35 proteins from the *Saccharomyces sensu stricto* group (36). Levels of sequence divergence among these proteins overlap the range of divergence detected among the prion proteins of the distantly related mammalian species. In agreement with previous reports demonstrating the higher rate of Sup35NM evolution in comparison to Sup35C (26), the amino acid sequences of the N, M, and C regions show, respectively, 94%, 87%, and 100% of identity between *S. cerevisiae* and *S. paradoxus* (separated by 5 million year evolutionary distance), and respectively, 77%, 72%, and 97% of identity between *S. cerevisiae* and *S. bayanus* (20 million year evolutionary distance). *S. bayanus* ORs region is shortened by one repeat compared to other two species (37-39) (Figure 1.6).

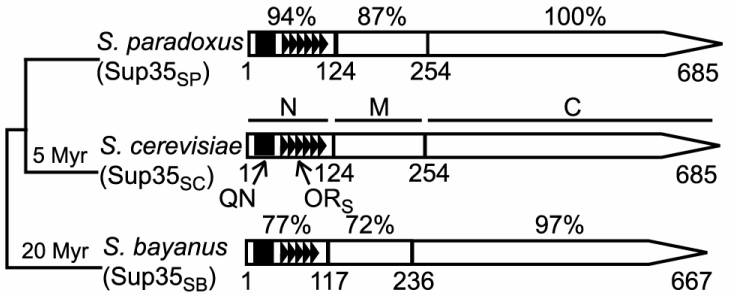


Figure 1.6 Structural and functional organization of the *S. sensu stricto* Sup35 proteins
SC, SP and SB refer to *S. cerevisiae*, *S. paradoxus* and *S. bayanus*, respectively. Percentage of amino acid identity to *S. cerevisiae* is shown for each region of *S. paradoxus* and *S. bayanus* proteins individually. For sequence alignment of the Sup35N regions, see Chapter 3, Figure 3.1. Designations are the same as Figure 1.3.

Previously, species barrier studies employing *Saccharomyces* Ure2 system have been performed. [URE3] prion of *S. cerevisiae* was efficient transmitted to *S. cerevisiae ure2Δ* cells expressing Ure2 of *S. paradoxus* or *S. bayanus*, which suggested that the homologous prion protein Ure2 from the closely related *Saccharomyces* species exhibits no species barrier (40). However, researchers disagreed on whether or not *S. paradoxus* Ure2 is capable of forming a prion at all (41, 42). Therefore, it remained unclear whether prion species barrier exists in yeast at the levels of sequence divergence that are comparable to those observed in mammals.

1.8 Objectives

The main goal of this work is to study whether or not there is a species barrier between the closely related yeast prion proteins, and if yes, what is the mechanism controlling the

species barrier in this system. As Sup35 divergence within the genera *Saccharomyces* system is very similar to that among mammalian prions, our work could shed light on mechanisms controlling cross-species prion transmission in mammals and help with preventing or possibly curing prion diseases.

CHAPTER 2

Overview of material and methods

2.1 Materials

2.1.1 Yeast strains

Yeast strains used and constructed in this study are listed in Appendix A. See following chapters for detailed descriptions and constructions.

With the exception of cytoduction recipients, all *S. cerevisiae* strains were isogenic [PSI^+] and [psi^-] derivatives of GT81 (31) of the following genotype: *MAT***a** (or *MATα*) *ade1-14$_{SC}$ his3 leu2 lys2 trp1 ura3*. Strains bearing the *sup35*Δ*::HIS3* transplacement on the chromosome and maintained alive by the *SUP35*-containing plasmids were constructed as described previously (31, 43) or introduced by plasmid shuffle in this study. The recipient strains for cytoduction were derivatives of 1B-D910 (*MAT***a** *ade1-14$_{SC}$ his3 leu2 trp1 ura3 cyh^R kar1-1* [*rho$^-$ psi$^-$ pin$^-$*]), kindly provided by A. Galkin (St. Petersburg University, Russia) and containing the *sup35*Δ*::HIS3* deletion on the chromosome with various *SUP35*-containing plasmids introduced by plasmid shuffle in this study. The *S. paradoxus* strain SP7-1D, kindly provided by G. Naumov (State Institute for Genetics and Selection of Industrial Microorganisms, Moscow, Russia), and *S. bayanus* strain FM361, kindly provided by M. Johnston (Washington University, St. Louis, MO), were the source of the *SUP35$_{SP}$* and *SUP35$_{SB}$* genes, respectively. The diploid *S. paradoxus*

strain GT749-1B of the following genotype: *MATα/MATa lys2/lys2 ura3-P2/ura3-P2,* has been constructed by G. Newnam in Chernoff lab, was the initial strain used for construction of *S. paradoxus* strains with appropriate markers. The haploid *S. bayanus* strains Su1A and Su1B (44) of genotype: *MAT***a** (or *MATα*), *ura3-1, hoΔ::KANMX4* were the initial strains used for construction of *S. bayanus* strains with appropriate markers.

2.1.2 Plasmids

Plasmids used and constructed in this study are listed and briefly described in Appendix B. All PCR-generated fragments were verified by sequencing. See following chapters for detailed descriptions and constructions.

2.1.3 Primers

Primers used in this study are listed in Appendix C.

2.1.4 Antibodies

The rabbit polyclonal Sup35NM antibodies were produced by Cocalico, Inc (45). The Sup35C antibodies were a gift of D. Bedwell (University of Alabama at Birmingham, Birmingham, AL). The Rnq1 antibodies were a gift of S. Lindquist (MIT, Cambridge, MA)

2.2 Methods

2.2.1 Molecular biology techniques

Standard protocols were used for DNA electrophoresis, restriction digestion and ligation (46). Enzymes were purchased from New England Biolabs and Invitrogen.

2.2.2 IsoPure Gel Extraction protocol

Fragments of DNA generated by restriction digestion or PCR reaction were separated using standard DNA electrophoresis (46). DNA bands corresponding to desired products were identified using a UV transilluminator (Fischer Biotech 312 nm Variable Intensity Transilluminator) and bands were excised from ethidium bromide (EtBr) – stained gels using a scalpel. Separation of DNA from gel was achieved using the IsoPure Gel Extraction Prep Kit.

2.2.3 *E. coli* plasmid DNA isolation

Quick plasmid DNA isolation was performed using the boiling prep method (46). Briefly, sterile wooden toothpicks were used to collect cells which were resuspended in STET buffer (5 % Triton X-100, 8 % sucrose, 20 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris-HCl, pH 8.0) with lysozyme added to a final concentration of 1mg/ml. Suspensions were boiled for 90 seconds, followed by centrifugation at 16,000 g for 15

minutes. The viscous pellets were removed using sterile toothpicks, and DNA in the remaining supernatant was precipitated with isopropanol at -20 ºC for 30 minutes. Precipitated DNA was collected by centrifugation at 16,000 g for 10 minutes, washed with 70 % ethanol, dried thoroughly, and was resuspended in TE+RNase (10 mM Tris-HCl, 1 mM EDTA, 0.1 mg/ml RNase, pH 7.4).

For isolation of purer plasmid DNA, sterile wooden toothpicks were used to collect cells from a quarter of the petri dish, and resuspend cells in 200 µl of Solution I (25 mM Tris-HCl, 10 mM EDTA, 0.9 % glucose, 2 mg/ml lysozyme, pH 8.0). Suspensions were incubated for 10 minutes, followed by adding 400 µl of Solution II (0.2 M NaOH, 1 % sodium dodecyl sulfate (SDS)). The mixtures were incubated on ice for 15 minutes before 300 µl of Solution III (5 M CH_3COONa – 3 M Na, 5 M acetate, pH 4.8) was added, and the mixtures were incubated on ice for another 30 minutes. Cell debris was pelleted at 16,000 g for 15 minutes. The supernatant was moved to another tube that contains 600 µl isopropanol and mixed well. The mixtures were incubated for 20 minutes. Precipitated DNA was collected by centrifugation at 16,000 g for 15 minutes, washed with 70 % ethanol, dried thoroughly, and resuspended 200 µl in TE+RNase. Suspensions were incubated at 37 ºC for 30 minutes, followed by adding 200 µl of 9 M lithium chloride (LiCl) and incubating at -20 ºC for 20 minutes. The mixtures were pelleted at 16,000 g for 10 minutes, and supernatant was moved to another tube containing 800 µl of 95 % ethanol. DNA was precipitated for 40 minutes, and collected at 16,000 g for 10 minutes. DNA pellet was washed by 70 % ethanol and dried thoroughly. Finally, dry pellets were resuspended in 30-50 µl of 10 mM Tris-HCl, pH 8.0.

2.2.4 Yeast and *E. coli* transformation procedures

All yeast transformations were performed according to lithium-treatment procedure (47), and plated onto synthetic selective medium except when selecting transformants conferring dominant drug resistance (hygromycin B, geneticin and nourseothricin), which was achieved by plating on YPD for 1-2 days followed by velveteen replica plating onto YPD with respective drug added. All *E. coli* transformations were prepared using chemically competent *E. coli* cells according to standard laboratory protocols (46), except for transforming HMS174 (pLysS) (see below, as described in *E.coli* expression system)

2.2.5 Standard yeast media and growth conditions

S. cerevisiae yeast cultures were grown at 30 ºC, and *S. paradoxus* and *S. bayanus* yeast cultures were grown at 25 ºC. Standard yeast media and standard procedures for yeast cultivation, phenotypic and genetic analysis, sporulation and dissection were used (48). Sporulating cultures were dissected using a micromanipulator Ergaval Series 10 from Carl Zeiss or The Singer MSM System 300. Synthetic media lacking histidine, adenine, leucine, tryptophan, lysine, or uracil are designated as –His, –Ade, -Leu, -Trp, -Lys and –Ura, respectively. In all cases when carbon source was not specifically indicated, 2 % glucose (Glu) was used. The synthetic medium containing 2 % galactose (Gal) or 2 % galactose and 2 % raffinose (Gal+Raf) instead of glucose was used to induce *GAL* promoter. Up to 150 uM copper sulfate ($CuSO_4$) was used for induce *CUP* promoter. Liquid cultures were grown with at least a 1/5 liquid/flask volumetric ration in a shaking

incubator (200-250 rpm). 0.3 mg/ml hygromycin B, 0.2 mg/ml geneticin and 0.1 mg/ml nourseothricin were added to YPD to select drug resistant colonies, respectively.

2.2.6 Yeast DNA isolation

Genomic DNA from yeast cultures was collected according to standard laboratory protocols (48). Briefly, cells were grown overnight, and collected by centrifugation at 2,000 g for 5 minutes. Cell pellets were resuspended in 0.5 ml of 1 M sorbital, 0.1 M EDTA, pH 7.5, and 40 µl of a 4 mg/ml lyticase was added. The suspensions were incubated at 37 ºC for overnight. Cells were collected by centrifugation at 2,000 g for 5 minutes, and were resuspended in 0.5 ml of 50 mM Tris-HCl, 20 mM EDTA, pH 7.4. 55 µl of 10 % SDS was added to suspensions and incubated at 65 ºC for 30 minutes. 0.2 ml of 5 M potassium acetate was added, and the mixtures were incubated on ice for 1 hour. Cell debris was collected by centrifugation at 16,000 g for 10 minutes, and the supernatant was moved to another tube containing 0.75 ml of isopropanol. DNA was precipitate at -20 ºC for 30 minutes before centrifugation at 16,000 g for 10 minutes. DNA was washed by 70 % ethanol, and dried thoroughly. The dry pellets were resuspended in 0.4 ml TE on a rotator (12 rpm) for 30 minutes before 2 µl of 10 mg/ml RNase was added, and the mixtures were incubated at 37 ºC for 30 minutes. 42.2 µl sodium acetate was added, followed by adding 0.84 ml 95 % ethanol. DNA was precipitate at -20 ºC for 30 minutes before centrifugation at 16,000 g for 10 minutes. DNA was washed by 70 % ethanol, and dried thoroughly. Finally, the dry pellets were resuspended in 50 µl of TE.

2.2.7 Yeast protein isolation and analysis

Protein isolation from yeast and centrifugation analysis were in accordance with previously published protocol (17). Briefly, yeast cultures were grown overnight, and cyclohexamide was added to a final concentration of 200 μg/ml 15 minutes before protein isolation. Cells were collected by centrifugation at 2,000 g for 5 minutes at 4 ºC, followed by washing cells with 300 μl of ice-cold lysis buffer I (50 uM Tris-HCl, 0.1mM EDTA, 1mM benzamidine, 2 μg/ml pepstastin A, 5 mM magnesium chloride ($MgCl_2$), 0.1 um Dithiotreitol (DTT), 2 mM phenylmethylsulfonyl fluoride (PMSF), 100 μg/ml RNase, 10 mM potassium chloride (KCl), 100 μg/ml cyclohexamide, 10 μg/ml Leupeptin, pH 7.5) or lysis buffer II (25 mM Tris-HCl, 100 mM sodium chloride (NaCl), 1 mM DTT, 10 mM EDTA, 2 mM PMSF and one tablet per 20 ml of Roche Complete (proteinase inhibitor cocktail), pH 7.5). Cells were resuspended in 300 μl of ice-cold lysis buffer, and about 300 μl of acid washed glass beads are added. Cells were lysed by glass beads three times by 1 minute vortex with at least 1 minute on ice between vortexes. Cell debris was removed by centrifugation at 2,000 g for 3 minutes to produce a "total lysate" fraction. Half of the total lysate was kept on ice, while the remaining was fractionated by centrifugation at 39,000 g (or higher) for 30 minute at 4 ºC. The supernatant was placed into a fresh tube, and the pellet was resuspended in an equal amount of the lysis buffer. SDS, glycerol, 2-mercaptoethanal and Tris-HCl (pH 6.8) were added to every sample up to final concentrations of 2.5 %, 10 %, 5 % and 25 mM, respectively. Resulting samples were boiled for 10 minutes to run on the standard SDS-polyacrylamide (SDS-PAGE) gel

or stored at -70 ºC. For performing the protein assays, gels were transferred onto Hybond ECL nitrocellulose membranes and reacted to the appropriate antibodies.

2.2.8 *E. coli* competent cells preparation

DH5α *E. coli* strain was inoculated to a 100 ml SOB (20 g/l Bactotryptone, 5 g/l Yeast Extract, 0.584 g/l NaCl, 0.186 g/l KCl and 5 ml/l 2 M Mg^{2+} was added after autoclaving). The culture was incubated at 37 ºC shaker until an OD_{550} reaches 0.45 to 0.55. Cells were incubated on ice for 15 minutes, and were collected by centrifugation at 2,000 g for 10 minutes at 4 ºC. Cells were resuspended into 33 ml RF1 (100 mM rubidium chloride (RbCl), 50 mM manganese chloride (MnCl), 30 mM Potassium acetate, 10 mM calcium Chloride ($CaCl_2$), 15 % Glycerol, pH 5.8). The suspension was incubated on ice for 45 minutes, and was collected by centrifugation at 2,000 g for 10 minutes at 4 ºC. Finally, cells were resuspended into 8 ml RF2 (10 mM morpholinopropanesulfonic acid (MOPS), 10 mM RbCl, 75 mM $CaCl_2$, 15 % Glycerol), and ready for use or moved to -70 ºC.

2.2.9 Confocal microscopy

Fluorescence microscopy was performed and images of the live yeast cells analyzed by using the LSM510 laser confocal microscope (Carl Zeiss, Inc., Jena, Germany) as described before (49). Briefly, an aliquot of growing yeast cells with plasmids which expressed green fluorescent protein (GFP) and red fluorescent protein (RFP) was placed on a glass slide, and sealed the cover slide with clear nail polish. An argon laser with an

excitation wavelength of 488 nm and pinhole size of 4.66 airy units was used for GFP detection, and a helium-neon laser with an excitation wavelength of 543 nm and pinhole size of 4.13 airy units was used for RFP detection.

2.2.10 DNA sequencing

All DNA sequencing was performed at the Nevada Genomics Center, Georgia Institute of Technology School of Biology Genomics Facility, FAME (Fundamental and Applied Molecular Evolution) center and MWG Biotech, Inc. DNA samples for sequencing were prepared using the IsoPure Gel Extraction Prep Kit, and eluted by water.

2.2.11 Plasmid shuffle and cytoduction

Plasmid shuffle and cytoduction were described previously (43, 50), and modification was specified in Chapters.

2.2.12 *E. coli* expression system

HMS174 (pLysS) (Novagen) was used as a host *E. coli* strain for pET20b expression system. The strain is a lysogen of bacteriophage λDE3, and carries a T7 RNA Polymerase gene under the control of the *lacUV5* promoter, the only promoter known to direct transcription of the T7 RNA polymerase gene, which is inducible by IPTG (isopropyl-β-thiogalactopyranoside). When IPTG is added to a growing culture of the λDE3 lysogen, expression of T7 RNA Polymerase is induced, and the polymerase

transcribes the target gene from pET20b vector. HMS174 strain is *recA* mutant that can help stabilize certain target genes. The pLysS plasmid encodes a natural inhibitor of the T7 RNA Polymerase. This inhibitor suppresses basal expression from the pET20b vector before expression is induced by the addition of IPTG.

HMS174 (pLysS) was inoculated in 5 ml of luria broth (LB) with 75 μg/ml chloramphenical (Cm) for overnight, and 100 μl of the culture was inoculated into 5 ml fresh LB+75 μg/ml Cm. The fresh culture was grown for 3 hours at 37 ºC before cells were collected by centrifugation at 2,000 g for 10 minutes at 4 ºC. The cell pellet was resuspended into 2.5 ml ice-cold 50 mM $CaCl_2$, followed by incubation on ice for 40 minutes. Cells were collected again by centrifugation at 2,000 g for 10 minutes at 4 ºC, and finally were resuspended into 500 μl of 50 mM $CaCl_2$. 100 μl of cells were transformed with pET20 series plasmid, and transformants were selected on LB+75 μg/ml Cm+100 μg/ml ampicillin (Amp).

Fresh transformants were inoculated into 10 ml LB+75 μg/ml Cm+100 μg/ml Amp for overnight, and the next day, an aliquot of culture was inoculated into 200 ml LB+75 μg/ml Cm+100 μg/ml Amp to an OD_{550} of 0.1 to 0.2. Culture was grown for additional several hours at 37 ºC to reach OD_{550} of 0.6 to 0.8 before 240 μl of 20% IPTG was added to induce expression for 4 hours. Cells were collected at 2,000g for 10 minutes at 4ºC, followed by protein purification or moved to -70ºC.

2.2.13 Purification of proteins with $(His)_6$ tag using Ni-NTA His-Bind Resins (Novagen)

The wet weight (gram) of the *E. coli* cells was measured on scale, and followed by completely resuspending the cells at room temperature in 5 ml BugBuster Protein Extraction Reagent (Novagen) per gram of wet cells. 1 µl Benzonase Nuclease (Novagen) was added per ml BugBuster Protein Extraction Reagent, and the cell suspension was rotated on a 12 rpm rotator for 20 minutes. The insoluble cell debris was moved by centrifugation at 16,000 g for 20 minutes at 4 °C, and the soluble extract was loaded onto Ni-NTA His-Bind Resin equilibrated with 1X Ni-NTA Bind Buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 10 mM imidazole, pH 8.0). For equilibration, 4 mL 1X Ni-NTA Bind Buffer was mixed with per mL 50 % Ni-NTA His-Bind slurry, and was settled by gravity before removing supernatant with a pipet. The lysate-Ni-NTA His-Bind mixture was loaded into a column, followed by washing the column with 8 mL 1X Ni-NTA Wash Buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 20 mM imidazole, pH 8.0) per mL 50 % Ni-NTA His-Bind slurry used. Finally, the protein was eluted with 2 mL 1X Ni-NTA Elute Buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 50 mM imidazole, pH 8.0) per mL 50% Ni-NTA His-Bind slurry used.

2.2.14 *In vitro* protein polymerization and cross-seeding

Protein was concentrated to 500 µM by the microcon filter devices (Amicon), and stored in 20 mM Tris-HCl, pH 8.0 with 6 M guanidine hydrochloride (GuHCl) and 300 mM

NaCl. For polymerization experiments (17), protein extracts were diluted 100-fold to 5 μM in 1 ml of 150 mM NaCl with 5 mM potassium phosphate, pH 7.4 and one tablet per 20 ml of Roche Complete, and incubated at room temperature with shaking at 12 rpm. Aliquots were taken after specified periods of time and mixed with SDS to the final concentration of 2 %. Half of each aliquot was boiled for 10 minutes to disaggregate polymers. Both boiled and non-boiled samples were run on SDS-PAGE and stained with Coomassie Brilliant Blue R-250. Polymerization was detected by a decrease in the proportion of protein entering the gel in the non-boiled versus boiled sample. Polymers not capable of entering the gel without boiling were usually seen at the start of the gel in the non-boiled samples.

CHAPTER 3*

Prion species barrier between the closely related yeast proteins is detected despite coaggregation

3.1 Introduction

In yeast, prion species barrier of Sup35 between highly divergent proteins from distantly related yeast genera *Saccharomyces* and *Pichia* or *Candida* coincided with the inability of heterologous prion domain to coaggregate *in vivo* and *in vitro* (21, 32-34). Homologs of prion protein Ure2 from the closely related *Saccharomyces* species exhibited no species barrier, although researcher disagreed on whether *Saccharomyces paradoxus* Ure2 is capable of forming a prion at all (40-42). Therefore, it remained unclear whether prion species barrier of Sup35 exists in yeast at the levels of sequence divergence that are comparable to those observed in mammals.

We have developed a new experimental system for studying prion species barrier that employs the closely related Sup35 proteins from the *Saccharomyces sensu stricto* group (36). Levels of sequence divergence among these proteins overlap the range of divergence detected among the prion proteins of the distantly related mammalian species. By using this system, we have demonstrated that closely related yeast proteins capable of coaggregation still exhibit a species barrier.

*** Materials of this chapter were published in part in Chen B, Newnam GP, Chernoff YO (2007) *Proc Natl Acad Sci USA* 104:2791-2796**

3.2 Materials and methods

3.2.1 Strains

Yeast strains used and constructed were described in Chapter 2, and listed in Appendix A.

3.2.2 Plasmids

The open reading frame (ORF) regions of the *SUP35* genes of *S. paradoxus* and *S. bayanus* ($SUP35_{SP}$ and $SUP35_{SB}$) were PCR-amplified by using primers with the extensions containing the restriction sites for *Bam*HI and *Sac*I, and used to replace the *S. cerevisiae SUP35* ($SUP35_{SC}$) ORF in the *Bam*HI-*Sac*I cut centromeric (*CEN)* vectors p316Sp-SUP35 (*URA3*) or p315Sp-SUP35HA3 (*LEU2*), kindly provided by J. Weissman (51). Resulting constructs, named respectively p316-PS-SUP35SP or p315-PS-SUP35SP for *S. paradoxus*, and p316-PS-SUP35SB or p315-PS-SUP35SB for *S. bayanus*, retained the endogenous *S. cerevisiae SUP35* (P_{SUP35}) promoter. Plasmids pYCH-U2 (*URA3*) (19) and pASB2 (*LEU2*) (43) bearing the $SUP35_{SC}$ gene were used as controls. Plasmid pRS316Gal-SUP35SB was constructed by cutting the *Bam*HI-*Sac*I fragment with $SUP35_{SB}$ ORF from p316-PS-SUP35SB and inserting it into pRS316GAL (52). *URA3* plasmid pmCUPNMsGFP, containing the *SUP35NM* region of *S. cerevisiae* ($SUP35NM_{SC}$) fused in frame to green fluorescent protein (GFP) ORF and placed under control of the copper-inducible (P_{CUP1}) promoter, as well as the original vector pmCUPsGFP were kindly provided by S. Lindquist (53). Plasmid pmCUP-SUP35SP was

constructed by replacing *GFP* ORF in pmCUPsGFP with the *Bam*HI-*Sac*I fragment of p316-PS-SUP35SP that contains $SUP35_{SP}$ ORF. Plasmid CEN-GAL-Sup35-RFP containing the NM region and a portion of the C region of $SUP35_{SC}$, fused in frame to red fluorescent protein (RFP) ORF and placed under control of the galactose-inducible (P_{GAL}) promoter, was described previously (50). The fusions of *S. paradoxus*, *S. bayanus* and *Pichia methanolica SUP35NM* ($SUP35NM_{SP}$, $SUP35NM_{SB}$ and $SUP35NM_{PM}$) to GFP were constructed by PCR-amplifying the respective *SUP35NM* regions and inserting them into pmCUPsGFP. Resulting plasmids were named pmCUP-NMSPsGFP, pmCUP-NMSBsGFP and pmCUP-NMPMsGFP, respectively. *TRP1* plasmids pFL39-CUP-NMSCsGFP, pFL39-CUP-NMSPsGFP, pFL39-CUP-NMSBsGFP and pFL39-CUP-PMsGFP were then constructed by moving the *Eco*RI-*Sac*I expression cassettes, each containing the P_{CUP1} promoter and respective chimeric gene originated from the respective pmCUP1-based vector, into the *CEN TRP1* vector pFL39 (54). The plasmids with chimeric *SUP35* genes were constructed as follows. First, the *SUP35N* regions of *S. paradoxus* and *S. bayanus* ($SUP35N_{SP}$ and $SUP35N_{SB}$) were produced by PCR-amplifying the corresponding fragments from the plasmids p316-PS-SUP35SP or p316-PS-SUP35SB, respectively with the primers containing the *Bam*HI and *Bgl*II extensions. These fragments were inserted into plasmid pmCUP1MCSC, that has been constructed by K. Gokhale in Chernoff lab and contains the *S. cerevisiae SUP35MC* ($SUP35MC_{SC}$) region under the copper-regulated P_{CUP1} promoter with *Bam*HI upstream of ORF. As this procedure generates a two amino acid insertion (RS) at the N and M boundary (after amino acid position 123 in *S. cerevisiae* and *S. paradoxus*, or 116 in *S. bayanus*), the $SUP35_{SC}$ gene with the same insertion has been reconstructed following the same

protocol, by using plasmid pRS315-SUP35, kindly provided by N. Riabinkova and S.G. Inge-Vechtomov, as a source of the *SUP35N* of *S. cerevisiae* ($SUP35N_{SC}$) region. At the next step, the *Bam*HI-*Sac*I fragment, containing full-size ORF of the chimeric or reconstructed *SUP35* gene, was cut out of the respective plasmid and inserted into the *CEN URA3* vector p316Sp-SUP35 (51) cut with the same enzymes, thus substituting for $SUP35_{SC}$ ORF. Resulting plasmids p316-PS-SUP35NSP-MCSC, p316-PS-SUP35NSB-MCSC and p316-PS-SUP35NSC-MCSC express the chimeric $SUP35N_{SP}MC_{SC}$, $SUP35N_{SB}MC_{SC}$, and $SUP35N_{SC}MC_{SC}$ genes, respectively, from the *S. cerevisiae* P_{SUP35} promoter. *E. coli* expression vector pET20b-Sup35NM producing the $Sup35NM_{SC}$ fragment tagged with the $(His)_6$ tag at the C-terminus, was described earlier (45). Analogous plasmids producing $Sup35NM_{SP}$-$(His)_6$ and $Sup35NM_{SB}$-$(His)_6$ were constructed by inserting the $SUP35NM_{SP}$ and $SUP35NM_{SB}$ fragments, PCR-amplified with extensions containing the *Nde*I and *Xho*I restriction sites from p316-PS-SUP35SP and p316-PS-SUP35SB respectively, into the *E. coli* plasmid pET20b (Novagen).

3.3 Results

3.3.1 Sequence alignment of the Sup35N regions of *Saccharomyces sensu stricto*

```
                    QN                                                  ORS
SP 1 MSDSNQGNNQQsYQQYgQNs NQQQGNNRYQGYQAYNAQsQ - PAGGYYQNYQGYSGYQQGs Y
SC 1 MSDSNQGNNQQNYQQYSQNGNQQQGNNRYQGYQAYNAQAQ - PAGGYYQNYQGYSGYQQGGY
SB 1 MSDpNQGNNQQNYQQYgQNf NQQQGNNkf QGYQAYNAQAQq PAGGYYQNpQGYaGYQQGGY

                                ORS
61 QQh- NPDAGYQQQYNPQGGYQQYNPQGGYQQQFNPQGGRGNYKNFNYNNNaQGYQAGFQPQSQG
61 QQY- NPDAGYQQQYNPQGGYQQYNPQGGYQQQFNPQGGRGNYKNFNYNNNLQGYQAGFQPQSQG
62 dQq fNP eAGYQQQYNaQG - - - - - - - - - GYQQQFNPQGGRGNYKsFNYs NNqQG f QAGFQPQSQG
```

Figure 3.1 Sequence alignment of the Sup35N regions of *Saccharomyces sensu stricto*

Figure 3.1 continued
Amino acid residues that are different in *S. paradoxus* (SP) or *S. bayanus*(SB), compared to *S. cerevisiae*(SC), are shown in the lower case and bold. Missing residues are indicated by dashes. (37-39), and Yeast Genome Database (http://www.yeastgenome.org/), and are confirmed by sequencing of our PCR-amplified clones. Differences in *SUP35* of *S. paradoxus* sequence between the strain SP7-1D used in our work and shown on this figure (37), and *S. paradoxus* strain accessible through Yeast Genome Database (39) are due to strain polymorphisms. Designations are the same as Figure 1.3 and 1.6.

3.3.2 Heterologous *S. sensu stricto* Sup35 proteins are capable of forming a prion in *S. cerevisiae*

The *SUP35* ORFs of *S. paradoxus* ($SUP35_{SP}$) and *S. bayanus* ($SUP35_{SB}$), placed onto a low-copy (*CEN*) shuttle plasmid under the endogenous *S. cerevisiae SUP35* promoter (P_{SUP35}), produced the respective Sup35 proteins at the same level as did the analogous *S. cerevisiae SUP35* ($SUP35_{SC}$) construct (data not shown), and conferred viability to the *S. cerevisiae* strain lacking the endogenous chromosomal $SUP35_{SC}$ gene.

To check whether *S. sensu stricto* Sup35 proteins can be turned into a prion state in the *S. cerevisiae* cell environment, we employed the UGA reporter allele *ade1-14* of *S. cerevisiae* ($ade1\text{-}14_{SC}$) and have shown that transient overproduction of $Sup35_{SP}$ or its NM-containing derivatives (Figure 3.2 A), or transient overproduction of $Sup35_{SB}$ (Figure 3.2 B) induced generation of the Ade^+ cells in the *S. cerevisiae* strain, lacking endogenous Sup35 and maintained alive by *CEN* plasmids bearing, respectively, $SUP35_{SP}$ or $SUP35_{SB}$.

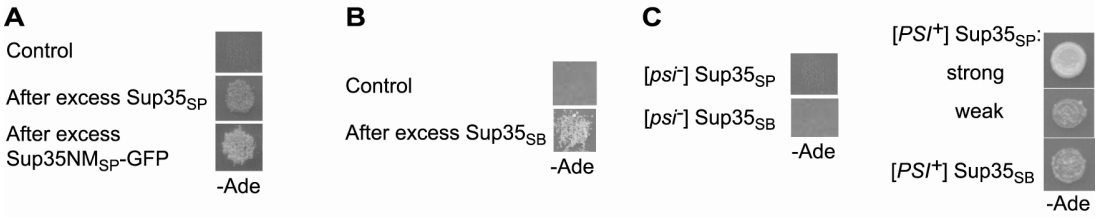


Figure 3.2 *S. paradoxus* and *S. bayanus* Sup35 proteins retain prion-forming abilities

(A-B) Transient overproduction of *S. paradoxus* Sup35 protein ($Sup35_{SP}$) or the $Sup35NM_{SP}$-GFP fusion protein (panel A), or transient overproduction of *S. bayanus* Sup35 protein ($Sup35_{SB}$) (panel B) induces prion formation in the [*psi*$^-$ *PIN*$^+$] *S. cerevisiae* strains bearing the $SUP35_{SP}$ (A) or $SUP35_{SB}$ (B) gene instead of $SUP35_{SC}$. Empty plasmids pmCUP-GFP (A) or pRS316GAL (B) were used as controls. Prion formation was detected by growth on –Ade medium following induction on P_{CUP1}-$SUP35_{SP}$ or P_{CUP1}-$SUP35NM_{SP}$-*GFP* constructs on the medium with 100 μM $CuSO_4$ (panel A), or P_{GAL}-$SUP35_{SB}$ construct on Gal medium (panel B). (C) $Sup35_{SP}$ generates both strong and weak prion variants, while $Sup35_{SB}$ generates only weak prion variants in *S. cerevisiae*, as judged from the efficiency of $ade1\text{-}14_{SC}$ suppression reflected by growth on –Ade. Note that both strong and weak variants of the $Sup35_{SP}$ prion show low mitotic stability (see Table 3.1). Plates were photographed after 5 (panel A), 8 (panel B) and 7 (panel C) days of incubation.

The resulting Ade$^+$ phenotype was curable by guanidine hydrochloride (GuHCl, data not shown) as typical of the [*PSI*$^+$] prion (17). Prion induction by overproduction was also detected in all possible heterologous combination of the "inducer" and "inducee" *S. sensu stricto* Sup35 proteins (data not shown). This observation is not surprising as even highly divergent Sup35NM region of *Pichia methanolica* is capable of inducing $Sup35_{SC}$ into a prion when overproduced (31, 33).

3.3.3 Suppression efficiency and mitotic stability of heterologous prion isolates in *S. cerevisiae*

In *S. cerevisiae*, multiple variants or "strains" of the endogenous [*PSI*$^+$] prion have been found (18). Like $Sup35_{SC}$, $Sup35_{SP}$ generated both strong and weak prion variants in *S. cerevisiae* as judged by growth (Figure 3.2 C) and color, but weak isolates were more abundant, and both strong and weak prion variants of $Sup35_{SP}$ accumulated 10-25% of [*psi*$^-$] colonies after 24-25 cell divisions in non-selective conditions (Table 3.1). Prion variants of $Sup35_{SB}$ were always characterized by weak suppression (Figure 3.2 C) and low stability (Table 3.1).

To identify the region of Sup35 responsible for low prion stability, we have constructed chimeric genes in which *SUP35N* region of $SUP35_{SC}$ was substituted with either $SUP35N_{SP}$ or $SUP35N_{SB}$. Chimeric protein with $Sup35N_{SB}$ continued to produce mitotically unstable prions, while chimeric protein with $Sup35N_{SP}$ produced prions of various mitotic stabilities, and some of these prion isolates were stable (Table 3.2). Therefore, decreased mitotic stability of heterologous prions is primarily determined by the differences in Sup35N region for $Sup35_{SB}$ but not for $Sup35_{SP}$.

Table 3.1 Mitotic stability of the heterologous Sup35 prion isolates in *S. cerevisiae*

Protein	Prion isolate	Number of cell divisions on non-selective medium	Number of colonies		
			[*PSI*$^+$]	[*psi*$^-$] (%)	Total
$Sup35_{SP}$	Strong	0	584	113 (16.2%)	697
		24.2	347	111 (24.2%)	458
	Weak	0	446	14 (3.0%)	460
		25.4	357	43 (10.8%)	400
$Sup35_{SB}$	Weak	0	471	41 (8.0%)	512
		23.2	270	58 (17.7%)	328

Initially, we checked 5 $Sup35_{SP}$ prion isolates and 17 $Sup35_{SB}$ prion isolates. Each $Sup35_{SP}$ prion isolate accumulated from 30% to 60% of [*psi*$^-$] colonies after approximately 40 or more generations in non-selective conditions, while each $Sup35_{SB}$ isolate accumulated from 4.3% to 50% of [*psi*$^-$] colonies after approximately 30 or more generations in non-selective conditions.

Two representative prion isolates of $Sup35_{SP}$ and one representative prion isolate of $Sup35_{SB}$ (also presented on Figure 3.2 C) were chosen for more detailed analysis as shown in Table 3.1. "Strong" and "weak" refer to intensity of growth on –Ade and color on YPD. In one version of the experiment, cultures were streaked out on YPD. After individual colonies have grown, they were cut off, cells were washed and counted. Numbers of cell divisions in non-selective conditions were determined from cell counts, under the assumption that each colony has originated from a single progenitor cell. Aliquot of each sample was plated onto YPD medium, and proportions of the [*PSI*$^+$] and [*psi*$^-$] cells in each initial colony were determined based on the phenotypes of colonies produced by these cells. Mosaics were counted as [*PSI*$^+$]. In the other version of the experiment, aliquots of the same initial colonies were plated on –Ade, and samples of the grown cultures were plated onto YPD to get individual colonies (0 divisions on non-selective medium). For each culture, average numbers of cell divisions and total counts of [*PSI*$^+$] and [*psi*$^-$] cells, based on analysis of 4 initial colonies are shown. For a given culture, results were generally homogenous among colonies.

Table 3.2 Mitotic stability of the Sup35 prion isolates generated by chimeric Sup35 protein

Protein	Prion isolate	Number	Colonies obtained in non-selective conditions		
			[*PSI*$^+$]	[*psi*$^-$] (%)	Total
$Sup35N_{SP}$-MC_{SC}	Strong	1	119	0 (0%)	119
		2	108	6 (5.3%)	114
		3	57	0 (0%)	57
		4	140	0 (0%)	140
		5	84	0 (0%)	84
		6	145	0 (0%)	145
		7	133	3 (2.2%)	135
	Weak	8	115	1 (0.9%)	116
		9	90	0 (0%)	90
		10	46	44 (48.9%)	90
		11	90	8 (8.3%)	98
		12	74	0 (0%)	74
		13	71	2 (2.7%)	73
$Sup35N_{SB}$-MC_{SC}	Strong	1	56	2 (3.4%)	58
	Weak	2	9	99 (91.7%)	108
		3	26	121 (82.3%)	147
		4	10	91 (90%)	101
		5	13	105 (89%)	118
		6	16	110 (87.3%)	126

All prion isolates were induced independently of each other. All cultures were grown for at least 30 or more cell divisions in non-selective conditions. Mosaic colonies (usually rare in stable [*PSI*$^+$] isolates) were counted as [*PSI*$^+$]. “Strong” and “weak” refer to intensity of growth on –Ade and color on YPD (as in Figure 3.2 C and Table 3.1).

3.3.4 Divergent Sup35 proteins co-aggregate in *S. cerevisiae*

$Sup35_{SC}$ protein is precipitated at high speed from the extracts of the prion-containing ([*PSI*$^+$]) strain, while the non-prion ([*psi*$^-$]) strain retains a significant fraction of $Sup35_{SC}$ in the soluble phase (17). Centrifugation analysis confirmed that proportion of the $Sup35_{SP}$ or $Sup35_{SB}$ protein in the aggregated state was increased in a culture containing

respective protein in the prion form, compared to the isogenic non-prion culture (data not shown).

In extracts of the *S. cerevisiae* [*PSI*$^+$] strain, bearing both endogenous *SUP35*$_{SC}$ and newly introduced *SUP35*$_{SB}$ or *SUP35*$_{SP}$, the Sup35-reacting material was shifted to the pellet, in contrast to the isogenic [*psi*$^-$] strain (Figure 3.3 A). This indicates that heterologous protein is precipitated together with the endogenous Sup35$_{SC}$ prion aggregates. Shift of Sup35$_{SB}$ could be visualized directly, as this protein is distinguishable by size from Sup35$_{SC}$. To visualize the Sup35$_{SP}$-based construct, we employed a chimeric protein composed of the N and M regions of Sup35$_{SP}$ fused to the green fluorescent protein (GFP). This protein, distinguishable by size from intact Sup35$_{SC}$, was also shifted to the insoluble phase together with Sup35$_{SC}$ in the [*PSI*$^+$] compared to the [*psi*$^-$] extracts (Figure 3.3 B).

Co-aggregation of the heterologous Sup35 proteins was also confirmed by fluorescence microscopy. We employed the Sup35NM fragments tagged with GFP or red fluorescent protein (RFP). These tagged proteins usually show diffuse fluorescence in the non-prion cells (as confirmed by us for the constructs based on Sup35NM$_{SP}$ or Sup35NM$_{SB}$) but generate cytologically detectable clumps in yeast cells bearing Sup35 in a prion state (17). The constructs producing GFP-tagged Sup35NM$_{SC}$, Sup35NM$_{SP}$ or Sup35NM$_{SB}$ were introduced into the strain containing endogenous Sup35$_{SC}$ in a prion form and bearing the plasmid that produces Sup35$_{SC}$-RFP. Green and red clumps co-localized with each other in all or most cells where both types of clumps were detected (Figure 3.3 C-E). In

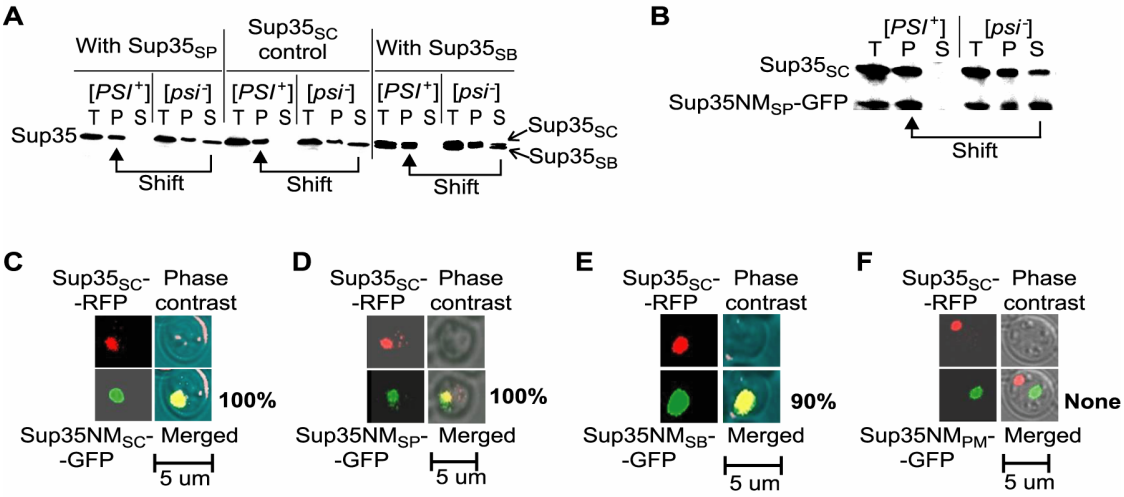


Figure 3.3 Co-aggregation of heterologous Sup35 proteins in *S. cerevisiae*
(A) The *S. cerevisiae* [*PSI*$^+$] strain simultaneously expressing both endogenous (Sup35$_{SC}$) and heterologous (Sup35$_{SP}$ or Sup35$_{SB}$) proteins shows all the Sup35-reacting material in the pellet (P) after centrifugation at 39,000 g, while the isogenic [*psi*$^-$] strain retains a fraction of Sup35 in the supernatant (S). Shift of Sup35$_{SB}$ to pellet can be monitored directly due to its lower molecular weight, compared to Sup35$_{SC}$. T- total lysate. (B) The chimeric Sup35NM$_{SP}$-GFP protein, expressed from the P_{CUP1} promoter in the presence of background levels (2 μM) of $CuSO_4$, shifts to pellet together with the endogenous Sup35$_{SC}$ protein in the [*PSI*$^+$] extract, in contrast to the [*psi*$^-$] extract. Designations are as in panel A. (C-F) The GFP-tagged NM fragments of Sup35$_{SC}$ (C), Sup35$_{SP}$ (D) and Sup35$_{SB}$ (E), but not the GFP-tagged Sup35NM fragment of *Pichia methanolica* (Sup35NM$_{PM}$-GFP, F) co-localize with the aggregated clumps of RFP-tagged Sup35$_{SC}$ in the *S. cerevisiae* [*PSI*$^+$] cells. GFP- and RFP-tagged constructs were expressed from the P_{CUP1} and P_{GAL} promoters, respectively, in the Gal+Raf medium supplemented with 150 μM $CuSO_4$. In each case, more than 100 cells containing both GFP and RFP aggregates were scored, and % of cells with co-localization is shown. Scale bars are indicated.

contrast, highly divergent Sup35NM of *Pichia methanolica*, tagged with GFP, never co-localized with Sup35$_{SC}$-RFP (Figure 3.3 F).

3.3.5 Species barrier in prion transmission between the divergent Sup35 proteins is detected despite co-aggregation

When an extra-copy of the homologous *SUP35* gene was introduced into the strain containing the respective protein in a prion form, it did not affect suppression of *ade1-14$_{SC}$*. In contrast, introduction of a plasmid with heterologous *SUP35* gene usually decreased or eliminated suppression, as detected by inhibition of growth on –Ade medium in the presence of such a plasmid (Figure 3.4 A). Suppression was restored on the medium not selective for the heterologous plasmid (data not shown), indicating that endogenous Sup35$_{SC}$ prion was not lost. Thus, heterologous Sup35 protein remained functional, despite its aggregation (see above, Figure 3.3).

In order to check whether the prion state can be transmitted between the heterologous Sup35 proteins, plasmid shuffle was performed. For this purpose, the [*PSI*$^+$] *sup35*Δ strain bearing *SUP35$_{SC}$* on a *CEN* plasmid has been individually transformed with *CEN* plasmids bearing *SUP35$_{SC}$*, *SUP35$_{SP}$* or *SUP35$_{SB}$* with a different marker. Transformation was followed by the loss of the original *SUP35$_{SC}$* plasmid. While shuffle for *SUP35$_{SC}$* to *SUP35$_{SC}$* exclusively produced [*PSI*$^+$] progeny, shuffle from *SUP35$_{SC}$* to *SUP35$_{SP}$* or *SUP35$_{SB}$* almost exclusively produced the [*psi*$^-$] progeny (Figure 3.4 A). As confirmed by centrifugation analysis, these [*psi*$^-$] colonies contained the Sup35$_{SP}$ or Sup35$_{SB}$ protein in the soluble phase, and did not restore the [*PSI*$^+$] state after reintroduction of the *SUP35$_{SC}$* plasmid, followed by the loss of *SUP35$_{SP}$* or *SUP35$_{SB}$* plasmid (data not shown). In the rare exceptional cases when cross-species conversion from Sup35$_{SC}$ to Sup35$_{SP}$ or

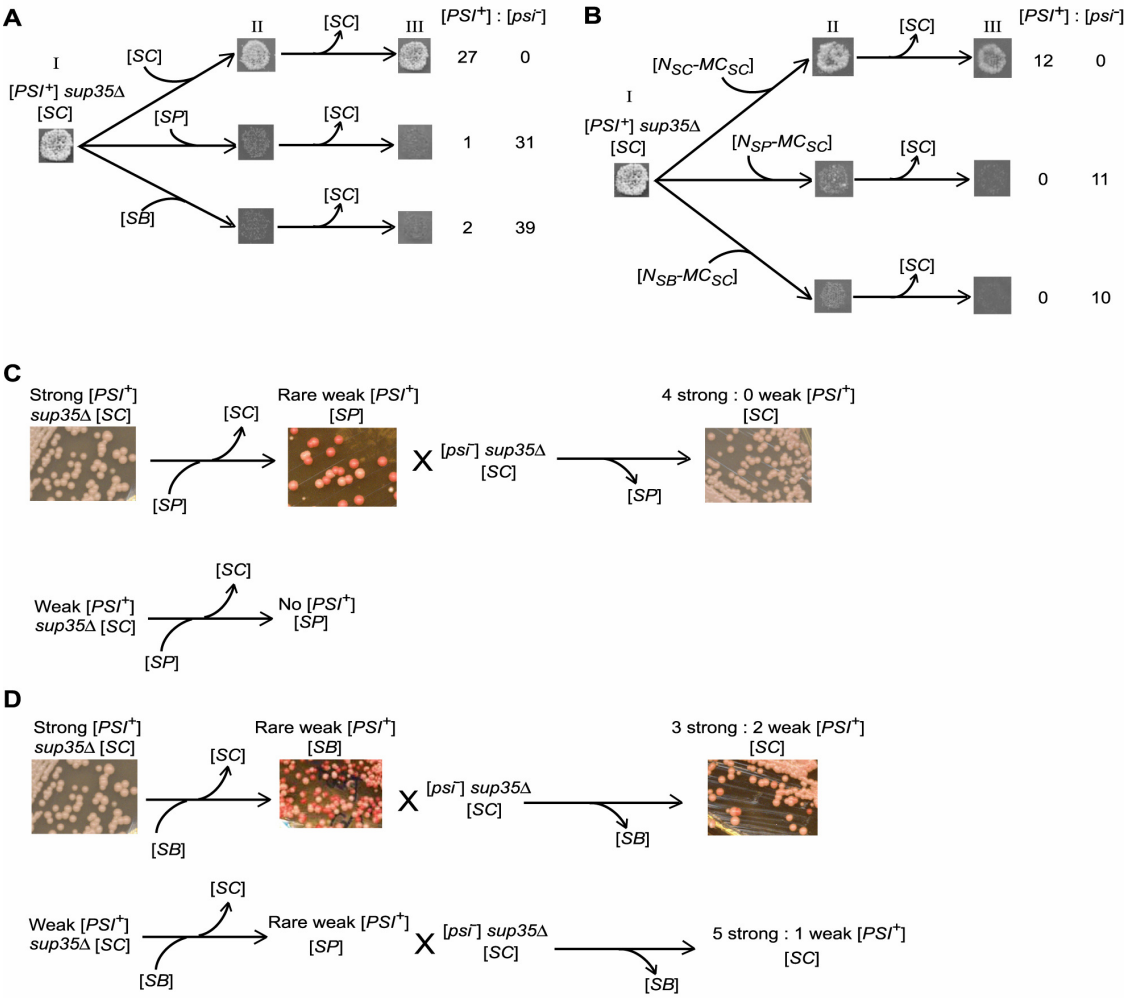


Figure 3.4 Prion species barrier between the closely related Sup35 proteins
Designations *SC*, *SP* and *SB* refer to the $SUP35_{SC}$, $SUP35_{SP}$ and $SUP35_{SB}$ genes, respectively. (A-B) Donor [*PSI*$^+$] *sup35*Δ strain with *SC* gene on the *CEN* plasmid, which grew on –Ade before the experiment (stage I), was transformed individually with *CEN* plasmids either bearing the intact *SC*, *SP* or *SB* genes (panel A), or containing the chimeric constructs composed of the *SUP35MC* region of *S. cerevisiae* (MC_{SC}) and *SUP35N* regions (*N*) of various origins (panel B). On panel B, reconstructed *SUP35* gene with $SUP35N_{SC}$ origin was used as a control. In contrast to intact or reconstructed $SUP35_{SC}$, heterologous genes (A) or chimeric genes with the heterologous *N* regions (B) inhibited suppression of $ade1\text{-}14_{SC}$ by [*PSI*$^+$], as judged by decreased growth on –Ade medium selective for both plasmids (stage II). After elimination of the original *SC* plasmid (stage III), all colonies with the new *SC* plasmid or reconstructed N_{SC}-MC_{SC} plasmid retained [*PSI*$^+$] while most or all colonies with *SP*, *SB* or chimeric plasmids containing N_{SP} or N_{SB} lost [*PSI*$^+$], as seen by growth/no growth on –Ade medium, respectively. Numbers of [*PSI*$^+$] and [*psi*$^-$] colonies obtained are given in each case. (C-D) Rare cross-species prion derivatives of $Sup35_{SP}$ or $Sup35_{SB}$, originated from plasmid shuffle from strong [*PSI*$^+$] *sup35*Δ strain with *SC* gene (as shown in panel A) exhibited weak suppression (as seen by pink

Figure 3.4 continued
color) and spontaneously produced [*psi*$^-$] (red) colonies, despite that the original *S. cerevisiae* [*PSI*$^+$] strain was strong (white) and stable. No cross-species prion derivatives of $Sup35_{SP}$ was generated from weak [*PSI*$^+$] *sup35*Δ strain with *SC* gene, and very similar prion derivates of $Sup35_{SB}$ was generated from the weak variant. After a cross to the isogenic [*psi*$^-$] strain bearing the $SUP35_{SC}$ plasmid, prion derivatives of $Sup35_{SP}$ and $Sup35_{SB}$ produced diploids that did not grow on –Ade selective for both plasmids, indicative of species barrier. After the loss of $SUP35_{SP}$ or $SUP35_{SB}$ plasmid, predominantly [*psi*$^-$] cultures were produced. Rare exceptional [*PSI*$^+$] isolates recovered from these cultures restored the initial characteristics of the strong *S. cerevisiae* [*PSI*$^+$] strain if obtained through $Sup35_{SP}$ (C), but some of the [*PSI*$^+$] isolates remained weak and unstable if obtained through $Sup35_{SB}$ (D). YPD plates were photographed after 3 days of incubation followed by refrigeration for 7 days. Numbers of strong and weak variants were given, respecitively.

$Sup35_{SB}$ occurred, it generated weak [*PSI*$^+$] strains with low mitotic stability (data not shown) similar to the majority class prions generated by these proteins in result of self-induction (see above), even if strong $Sup35_{SC}$ prion variant was used as the initial prion source.

Moreover, the [*PSI*$^+$] state was not transmitted by shuffle from $SUP35_{SC}$ to the chimeric constructs containing the *SUP35N* regions of *S. paradoxus* or *S. bayanus* and *SUP35MC* region of *S. cerevisiae* (Figure 3.4 B). This shows that the Sup35N region is responsible for the species barrier.

Species barrier was also confirmed when diploid [*PSI*$^+$] strain homozygous by $ade1\text{-}14_{SC}$ and heterozygous by *sup35*Δ*::HIS3* has been transformed with a *CEN* plasmid containing $SUP35_{SC}$, $SUP35_{SP}$ or $SUP35_{SB}$, sporulated and dissected. All *sup35*Δ [$SUP35_{SC}$] spore clones remained [*PSI*$^+$], while all *sup35*Δ spore clones with the $SUP35_{SP}$ or $SUP35_{SB}$ plasmid became [*psi*$^-$] (Table 3.3).

Table 3.3 Confirmation of the prion species barrier by tetrad analysis

Plasmid	His^+Ura^+ (*sup35*Δ) spores		
	Ade^+ ([*PSI*$^+$])	Ade^- ([*psi*$^-$])	Total
URA3 SUP35$_{SC}$	12	0	12
URA3 SUP35$_{SP}$	0	8	8
URA3 SUP35$_{SB}$	0	10	10

The isogenic diploid [*PSI*$^+$] strains homozygous by *ade1-14*$_{SC}$, heterozygous by *sup35*Δ*::HIS3* and containing a *CEN URA3* plasmid with *SUP35*$_{SC}$, *SUP35*$_{SP}$ or *SUP35*$_{SB}$, were sporulated and dissected. His^+ Ura^+ spores, bearing the *sup35*Δ*::HIS3* disruption and maintained alive by the plasmid were checked for the presence of [*PSI*$^+$]. Only *SUP35*$_{SC}$ plasmid maintained the [*PSI*$^+$] state.

Prions generated by $Sup35_{SP}$ and $Sup35_{SB}$ were frequently lost during transformation that complicated use of the above mentioned techniques for these strains. To check whether prion state is transmitted from $Sup35_{SP}$ or $Sup35_{SB}$ to $Sup35_{SC}$, we mated the [*PSI*$^+$] *sup35*Δ strains bearing a *SUP35*$_{SP}$ or *SUP35*$_{SB}$ plasmid to the isogenic [*psi*$^-$] *sup35*Δ strains bearing either homologous or heterologous (*SUP35*$_{SC}$) gene on a plasmid with a different marker. Resulting diploids were cured of the original plasmid and checked for the presence of [*PSI*$^+$]. [*PSI*$^+$] cells were obtained in heterologous combinations, although with at least several fold lower frequency than in homologous combinations (data not shown). Remarkably, while $Sup35_{SB}$ prions (generated from cross-species conversion from strong and weak origins) produced a variety of $Sup35_{SC}$ prion isolates of different stringencies, suggesting the variant-specific patterns were not maintained through the $Sup35_{SB}$ protein, the $Sup35_{SP}$ prion usually produced strong and stable $Sup35_{SC}$ prion isolates (Figure 3.4 C-D). This phenomenon formally resembles so-called “adaptation” of the heterologous prion “strains” after conversion of prion state to the host protein in the mammalian systems (5).

Finally, we compared efficiencies of prion transmission between all combinations of the *S. sensu stricto* Sup35 proteins used in this work in one and the same type of assay by employing cytoplasm transfer, or cytoduction (3). Cytoplasm was transferred from each of the [*PSI*$^+$] *sup35*Δ donor strains with different *SUP35* genes to the set of recipient karyogamy-deficient [*psi*$^-$] *sup35*Δ strains with different *SUP35* genes. The [*PSI*$^+$] transmission was highly efficient in each homologous combination but very inefficient in most heterologous combinations, specifically from $Sup35_{SC}$ to $Sup35_{SP}$ or $Sup35_{SB}$, from $Sup35_{SP}$ to $Sup35_{SB}$, and from $Sup35_{SB}$ to $Sup35_{SP}$ (Table 3.4). Interestingly, the prion state was transferred with high frequency from $Sup35_{SP}$ to $Sup35_{SC}$, and with only moderately decreased frequency from $Sup35_{SB}$ to $Sup35_{SC}$, indicating that prion species barrier is not completely symmetric. Cross-species prion conversion detected in these combinations by cytoduction was certainly higher than one detected by mating and shuffle assay (see above), although the same prion derivates of $Sup35_{SP}$ and $Sup35_{SB}$ obtained through cross-species plasmid shuffle were used in both assays. This could be due to either different genotype of the cytoduction recipient strains, or more likely, different experimental design. In cytoduction assay, number of cell divisions in non-selective conditions both before and after cytoplasm transfer was minimized, thus effectively eliminating the possibility of the spontaneous loss of prion state. Therefore, we believe that cytoduction provides a more accurate assessment of the efficiency of cross-species conversion. Observed asymmetry of prion species barrier in yeast resembles the situation described in some mammalian systems (10).

Table 3.4 Transmission of prion state by cytoduction

[*PSI*$^+$] donor	[*psi*$^-$] recipient	Cytoductants		
		[*PSI*$^+$] (%)	[*psi*$^-$]	Total
SUP35$_{SC}$	*SUP35*$_{SC}$	64 (100%)	0	64
	SUP35$_{SP}$	7 (13.0%)	47	54
	SUP35$_{SB}$	1 (2.1%)	46	47
SUP35$_{SP}$	*SUP35*$_{SC}$	43 (95.6%)	2	45
	SUP35$_{SP}$	39 (97.5%)	1	40
	SUP35$_{SB}$	1 (3.8%)	25	26
SUP35$_{SB}$	*SUP35*$_{SC}$	33 (76.7%)	10	43
	SUP35$_{SP}$	2 (6.9%)	27	29
	SUP35$_{SB}$	39 (97.5%)	1	40

The donor [*PSI*$^+$] *sup35*Δ strains with a *SUP35* gene of *S. cerevisiae*, *S. paradoxus* or *S. bayanus* were grown on –Ade medium to minimize the spontaneous loss of [*PSI*$^+$] and mated on YPD medium individually to each representative of the set of cytoduction recipient [*psi*$^-$] *sup35*Δ strains bearing a *SUP35* plasmid with a different marker. After mating, cells were plated onto the medium with ethanol, glycerol and cycloheximide, selective for cytoductants. Resulting colonies were analyzed for the presence of [*PSI*$^+$] by growth on –Ade medium. Numbers of [*PSI*$^+$] and [*psi*$^-$] cytoductants are given. Exceptional Ura$^+$ cytoductants in which the *SUP35* plasmid was transferred from the donor were excluded from analysis. For the [*PSI*$^+$] *SUP35*$_{SC}$ donor, the same results were obtained with the other (weak) prion variant, as well as in the version of the experiment where the donor strain was grown on non-selective conditions (data not shown). The control [*psi*$^-$] donor strains did not produce Ade$^+$ cytoductants (data not shown).

3.3.6 Cross-seeding and cross-inhibition of each other's polymerization by the divergent Sup35 proteins *in vitro*

In vitro polymerization of a purified Sup35NM fragment has been routinely used to study prion properties of Sup35 (55-57). Our data (Figure. 3.5) show that Sup35NM$_{SP}$ and Sup35NM$_{SB}$ are spontaneously polymerized *in vitro* in the non-denaturing conditions even more rapidly than Sup35NM$_{SC}$.

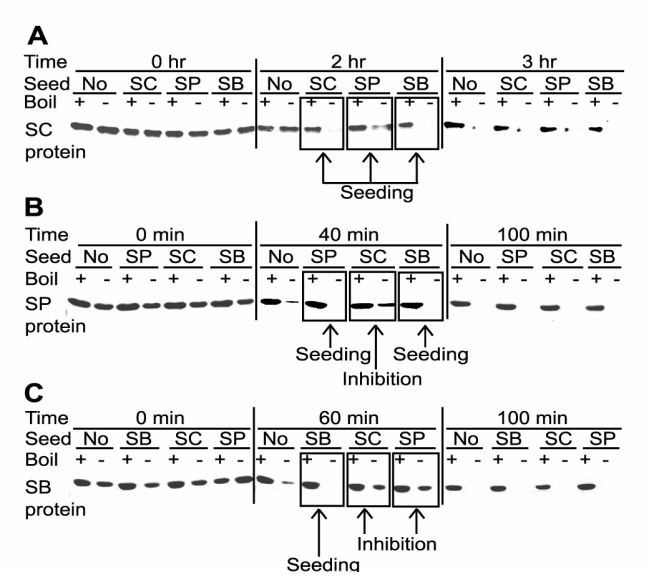


Figure 3.5 *In vitro* polymerization of the *S. sensu stricto* Sup35NM protein fragments

The purified $(His)_6$-tagged Sup3NM regions of *S. cerevisiae* ($Sup35NM_{SC}$ or SC, panel A), *S. paradoxus* ($Sup35NM_{SP}$ or SP, panel B), and *S. bayanus* ($Sup35NM_{SB}$ or SB, panel C) spontaneously polymerize in the non-denaturing conditions after a certain lag period, as detected by a decrease of the monomeric fraction remaining soluble in SDS and capable of entering the SDS-PAGE gel without boiling. Boiled samples where all protein enters the gel are shown in each case as controls. Addition of preformed polymers at the ratio of 1:20 leads to the following results: $Sup35NM_{SC}$ promotes polymerization of $Sup35NM_{SC}$ (A), but delays polymerization of both $Sup35NM_{SP}$ (B) and $Sup35NM_{SB}$ (C); $Sup35NM_{SP}$ promotes polymerization of both $Sup35NM_{SC}$ (A) and $Sup35NM_{SP}$ (B) but delays aggregation of $Sup35NM_{SB}$ (C); $Sup35NM_{SB}$ promotes polymerization of $Sup35NM_{SC}$ (A), $Sup35NM_{SP}$ (B) and $Sup35NM_{SB}$ (C).

For each of these three proteins, lag period of polymerization reaction was decreased by addition of the preformed homologous Sup35NM polymers ("seeds") at a 1:20 ratio**.** Likewise, polymerization of $Sup35NM_{SC}$ was accelerated by the heterologous preformed polymers of $Sup35NM_{SP}$ or $Sup35_{SB}$ (Figure 3.5 A), confirming that divergent Sup35NM domains of *S. sensu stricto* interact to each other *in vitro* as well as *in vivo*. Polymerization of $Sup35NM_{SP}$ was accelerated by the preformed $Sup35NM_{SB}$ "seed", but delayed by the preformed $Sup35NM_{SC}$ "seed" (Figure 3.5 B), while polymerization of

Sup35NM$_{SB}$ was delayed by the preformed heterologous polymers of Sup35NM$_{SC}$ or Sup35NM$_{SP}$ (Figure 3.5 C). Thus, species barrier between the *S. sensu stricto* proteins is detected *in vitro*, although not in all reciprocal combinations. Moreover, *in vitro* assay confirms the asymmetry of the species barrier between Sup35$_{SC}$ and other *S. sensu stricto* Sup35 protein, detected by cytoduction.

3.4 Discussion

3.4.1 Potential mechanism of prion species barrier at low levels of sequence divergence

While mammalian PrP proteins with 90% or higher amino acid identity exhibit species barrier (5), previous reports of yeast prion species barrier dealt with highly divergent PrDs retaining only 30-40% of identity (26). By detecting prion species barrier in yeast at the levels of identity up to 94%, we demonstrate that yeast prion conversion also requires nearly a precise correspondence of amino acid sequences.

While inefficient cross-species conversion between proteins with highly divergent PrDs was apparently due to their inability to co-aggregate both *in vivo* and *in vitro* (21, 32-34), yeast proteins with closely related PrDs exhibit species barrier (Figure 3.4) even in combinations for which efficient *in vivo* co-aggregation was observed (Figure 3.3). *In vitro*, purified *S. sensu stricto* PrD-containing fragments either cross-seed or inhibit each other's polymerization, depending on the combination (Figure 3.5). Either effect is hard

to explain by a mechanism which would not involve direct interactions between heterologous proteins. Taken together, our data clearly demonstrate that mechanical association of the heterologous proteins is not sufficient for efficient transmission of a prion state. This also agrees with the previous observation that heterologous mammalian PrPs can bind each other without efficient conversion to the proteinase-resistant state, and that heterologous binding may inhibit homologous conversion (58). Therefore, in both yeast and mammalian systems prion species barrier between closely related proteins appears to be controlled at levels other than simple co-aggregation.

As heterologous co-aggregation fails to efficiently produce a heritable prion state, it appears that sequence divergence impairs conformational transition. Difference by only a few ($Sup35NM_{SC}$ and $Sup35NM_{SP}$ PrDs) or even by one ((13), and see Chapter 4, Figure 4.7 for details) amino acid residue is sufficient for prion species barrier in some assays, suggesting that interactions between certain specific amino acid residues play a crucial role in achieving the maximal efficiency of the conformational transition. Further investigation of the prion species barrier between the closely related PrDs helps us to identify these positions at least in case of *S. cerevisiae* and *S. paradoxus* combination (see Chapter 4 for details).

Most heterologous prions generated by the *S. paradoxus* and *S. bayanus* proteins in the *S. cerevisiae* cell environment were weak and exhibited a high frequency of mitotic loss (Figure 3.2 C and Table 3.1). Propagation of yeast prions is thought to occur via generation of new polymerization “seeds” in result of chaperone-mediated fragmentation

of amyloid polymers (15), while mitotically unstable prions apparently are defective in their ability to be fragmented by the chaperones (43, 50, 59). It is an intriguing possibility that polymers generated by heterologous PrDs are not capable of efficient fragmentation in the *S. cerevisiae* cell environment as they are not adjusted to the levels or activities of the *S. cerevisiae* chaperones. Indeed, $Sup35_{SB}$ PrD has a shortened OR region (Figure 3.1 and Figure 1.6), previously implicated in control of prion fragmentation and propagation (25). Moreover, combination of *S. paradoxus* Sup35N region with *S. cerevisiae* Sup35MC increases prion mitotic stability (Table 3.2), in agreement with the observation that Sup35M influences interactions with the disaggregating chaperone Hsp104 (60). Interestingly, prions generated by a chimeric Sup35 protein with *Pichia* PrD also exhibited low mitotic stability (31, 33) and decreased sensitivity to Hsp104 (33). Defective prion propagation in a heterologous cell environment may represent an additional mechanism contributing to prion species barrier, but can not explain it completely, as restoration of prion mitotic stability in chimeric *S. paradoxus* – *S. cerevisiae* constructs did not eliminate the barrier (Figure 3.4 B).

Cross-inhibition of the *in vitro* Sup35 polymerization in some combinations by a small proportion (about 5 %) of the preformed heterologous "seed" suggests that the spontaneously arisen fraction of polymerization-proficient Sup35NM is initially very small. Remarkably, asymmetric patterns of *in vitro* cross-inhibition generally resemble asymmetry of the species barrier observed in the cytoduction assay *in vivo* (Table 3.4), as $Sup35NM_{SC}$ inhibits polymerization of $Sup35NM_{SP}$ or $Sup35NM_{SB}$, but not *vice versa* (Figure 3.5). The only exception was the *S. paradoxus* / *S. bayanus* combination

exhibiting a strong species barrier in both directions *in vivo* (Table 3.4) but only in one direction *in vitro* (Figure 3.5). This may reflect the difference in protein ratios between the *in vivo* and *in vitro* systems, and/or involvement of cell components other than Sup35 in the *in vivo* species barrier.

Overall, our data establish a yeast model for studying the mechanism of prion species barrier at low levels of sequence divergence, and pave the way for understanding the molecular processes responsible for this phenomenon.

3.5 Conclusions

Species barrier in prion transmission between the closely related Sup35 proteins is detected.

Closely related heterologous Sup35 proteins co-aggregate *in vivo*.

Closely related heterologous seed may inferfere with amyloid formation *in vitro*.

Species-specificity of prion transmission is controlled at the level of conformational transition rather than co-aggregation.

Prion domain is responsible for species barrier.

CHAPTER 4

Mapping of the Sup35 modules responsible for the species barrier

4.1 Introduction

We have previously demonstrated that Sup35N (prion domain) determines the prion "species barrier" among the *Saccharomyces sensu stricto* species ((1), see above Chapter 3, Figure 3.4 B for details).

In *S. cerevisiae*, Sup35N includes two regions that have previously been demonstrated to influence prion formation and propagation, namely QN-rich stretch (QN), located between the amino acid (aa) positions 6 and 40, and the region consisting of 5.5 imperfect oligopeptide repeats of the consensus sequence PQGGYQQ-YN (ORs), located between aa positions 41 and 97 (15). Sup35N domains of *S. paradoxus* and *S. bayanus* (Figure 4.1) show respectively 94% and 77% of aa identity to *S. cerevisiae* and maintain both QN and ORs regions despite divergence of their specific sequences, with one repetitive unit of ORs region missing in case of *S. bayanus* ((1, 37-39), see above Chapter 1, Figure 1.6 and Chapter 3, Figure 3.1 for details). Convenient location of the conserved recognition sites for restriction endonucleases *Hin*dIII and *Pfl*MI near the ends of the QN and ORs regions respectively (see Materials and methods, and Figure 4.2) enabled us to divide the Sup35N-coding region of the *SUP35* gene into 3 exchangeable modules, designated as I, II and III (Figure 4.1). Module I includes most of QN-stretch, including the whole region 6-28 with the maximal percentage of QN-residues, which is within the

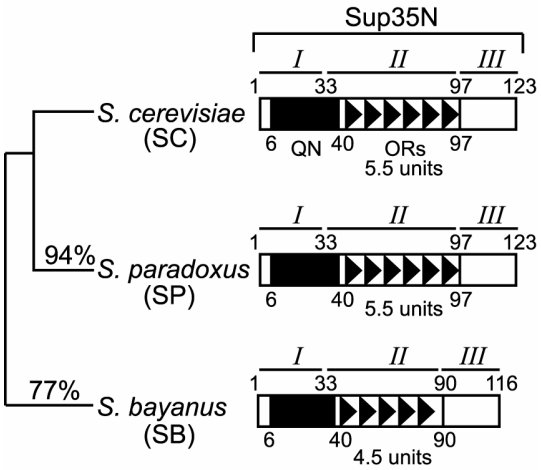


Figure 4.1 Structure and comparison of the Sup35N regions (prion domains) from different *Saccharomyces* species
Numbers correspond to amino acid positions. I, II and III refer to fragments of the Sup35N region, which have been exchanged in the chimeric constructions. QN and ORs refer to the QN-rich stretch and oligopeptide repeats, respectively. *SC – S. cecevisiae*, *SP- S. paradoxus*, *SB - S. bayanus*. The percentages of amino acid identity to $Sup35N_{SC}$ are shown.

region responsible for the species barrier in the *Saccharomyces-Candida* combination (21), and within which single aa substitutions with an anti-prion effect were detected (21, 51). Module II includes the very end of QN and the whole region of ORs, while module III includes the remaining portion of Sup35N that does not exhibit any specific sequence pattern.

In order to determine which specific region of Sup35N is responsible for the barrier, we have constructed a set of chimeric *SUP35N* genes as described below. After the responsible regions were located into a single module of *SUP35N*, which turned out to be different modules for different systems, the essential amino acid position was found using site-direct mutagenesis.

4.2 Materials and methods

4.2.1 Strains

Yeast strains used and constructed were described in Chapter 2, and listed in Appendix A.

4.2.2 Plasmids

The recognition site for restriction endonuclease *Hin*dIII between modules I and II is conserved among the three *Saccharomyces* species, and another *Hin*dIII recognition site is present within the *SUP35M* of *S. cerevisiae* (close to *SUP35N* and *SUP35M* boundary). The plasmids p316-PS-SUP35NSC-MCSC, p316-PS-SUP35NSP-MCSC and p316-PS-SUP35NSB-MCSC ((1), see Materials and methods of Chapter 3 above for details) were digested with *Hin*dIII, and small inserts containing modules (II-III) of different origins and the very beginning of *SUP35M* of *S. cerevisiae*, and large vectors containing endogenous promoter of the *S. cerevisiae SUP35* gene, module I of different origins, the rest portion of *SUP35M* and the complete *SUP35C* of *S. cerevisiae* were generated. The chimeric Sup35N domains containing exchanged modules I and (II-III) were obtained by ligating inserts with vectors obtained from a different digestion (Figure 4.2 A). All chimeric *SUP35N* domains constructed as described here and further were verified by sequencing.

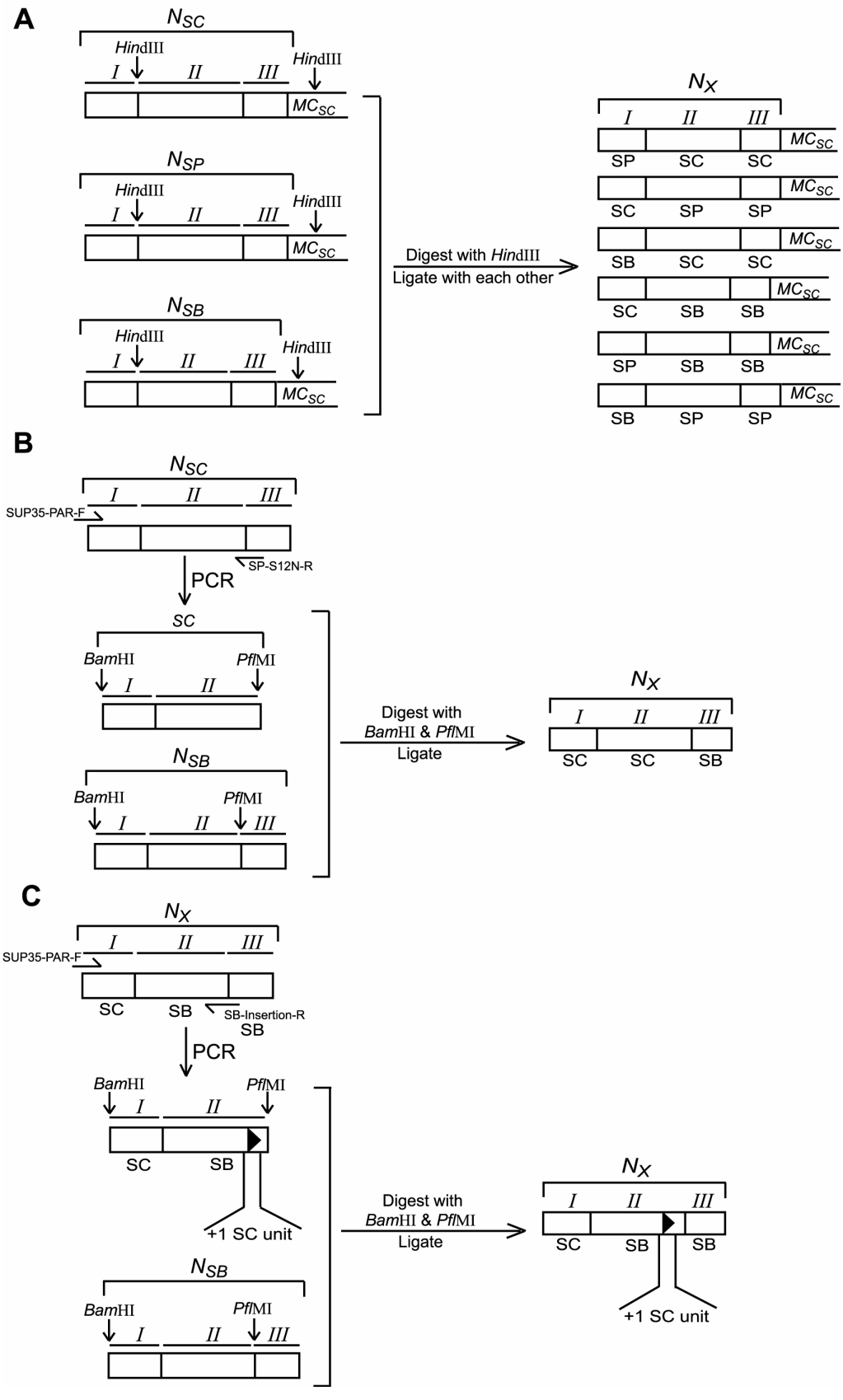

A
N_{SC}
HindIII
I
II
III
HindIII
MC_{SC}
N_{SP}
N_{SB}
Digest with HindIII
Ligate with each other
N_X
SP SC SC
SC SP SP
SB SC SC
SC SB SB
SP SB SB
SB SP SP
B
SUP35-PAR-F
SP-S12N-R
PCR
SC
BamHI
PflMI
Digest with
BamHI & PflMI
Ligate
SC SC SB
C
SB-Insertion-R
SC SB
+1 SC unit
SC SB SB

Figure 4.2 continued

Figure 4.2 Strategy used to construct plasmids with chimeric Sup35 prion domains N and MC refer to *SUP35N* and *SUP35MC* regions, respectively. SC, SP and SB refer to *S. cerevisiae*, *S. paradoxus* and *S. bayanus*, respectively. I, II and III refer to modules of Sup35 prion domain. X refers to a chimeric origin. The recognition sites for restriction endonucleases *Hin*dIII, *Bam*HI and *Pfl*MI are shown. Primers SUP35-PAR-F, SP-S12N-R and SB-insertion-R (Appendix C) are aligned with templates. "+1 SC" unit indicates the *S. bayanus* region II with one extra repetitive unit of *S. cerevisiae* origin (triangle) added.

The recognition site for restriction endonuclease *Pfl*MI between modules II and III is conserved among the three *Saccharomyces* species. *Bam*HI is present upstream of *SUP35* of different origins. Regions encompassing modules (I-II) of *S. cerevisiae* with *Bam*HI added upstream and *Pfl*MI added downstream by primers SUP35-PAR-F and SP-S12N-R (Appendix C) were PCR-amplified from p316-PS-SUP35NSC-MCSC ((1), see Materials and methods of Chapter 3 above for details). This fragment was digested with *Bam*HI and *Pfl*MI and used as an insert, while plasmid p316-PS-SUP35NSB-MCSC ((1), see Materials and methods of Chapter 3 above for details) was digested with the same restriction endonucleases and used as a vector. Ligation produced the plasmid with chimeric *SUP35N* domain containing only module III from *S. bayanus* and modules (I-II) from *S. cerevisiae* (Figure 4.2 B).

Regions encompassing module I of *S. cerevisiae* in conjunction with module II of *S. bayanus* were PCR-amplified from one of the chimeric constructs with module I of *S. cerevisiae* and modules (II-III) of *S. bayanus* (generated as described before). *Bam*HI was added upstream to the PCR fragment by primer SUP35-PAR-F (Appendix C), and an addition of one *S. cerevisiae* repetitive unit with *Pfl*MI were added downstream by primer

SB-Insertion-R (Appendix C). This fragment was digested with *Bam*HI and *Pfl*MI and used as an insert, while plasmid p316-PS-SUP35NSB-MCSC ((1), see Materials and methods of Chapter 3 above for details) was digested with the same restriction endonucleases and used as a vector. Ligation produced the construct containing module I of *S. cerevisiae* in conjunction with modules (II-III) of *S. bayanus* with the addition of one *S. cerevisiae* repetitive unit to the ORs region, which restoring its length to the size equal to *S. cerevisiae* (Figure 4.2 C).

Regions encompassing module I of *S. paradoxus* and modules (II-III) of *S. cerevisiae* with *Bam*HI added upstream and *Sac*I-*Bgl*II added downstream by primers SP-S12N-F#2 and NSC-R-BglII-SacI (Appendix C) were PCR-amplified from a chimeric construct with module I of *S. paradoxus* and modules (II-III) of *S. cerevisiae* (generated as described before). The S12N substitution was introduced through PCR-amplification by the forward primer (Figure 4.3 A). This fragment was digested with *Bam*HI and *Sac*I, and was inserted into pBSKII(+) (Stratagene) digested with the same restriction endonucleases. Module I of *S. paradoxus* with S12N substitution and modules (II-III) of *S. cerevisiae* were sequenced, and removed by *Bam*HI and *Bgl*II digestion. The chimeric *SUP35N* region with S12N substitution was fused to *SUP35M* and *SUP35C* of *S. cerevisiae*, and moved under endogenous promoter of the *S. cerevisiae SUP35* gene as described previously ((1), see Materials and methods of Chapter 3 above for details).

SUP35N of *S. cerevisiae* with *Bam*HI added upstream and *Sac*I-*Bgl*II added downstream by primers NSC-MCSC(N12S) and NSC-R-BglII-SacI (Appendix C) was PCR-amplified

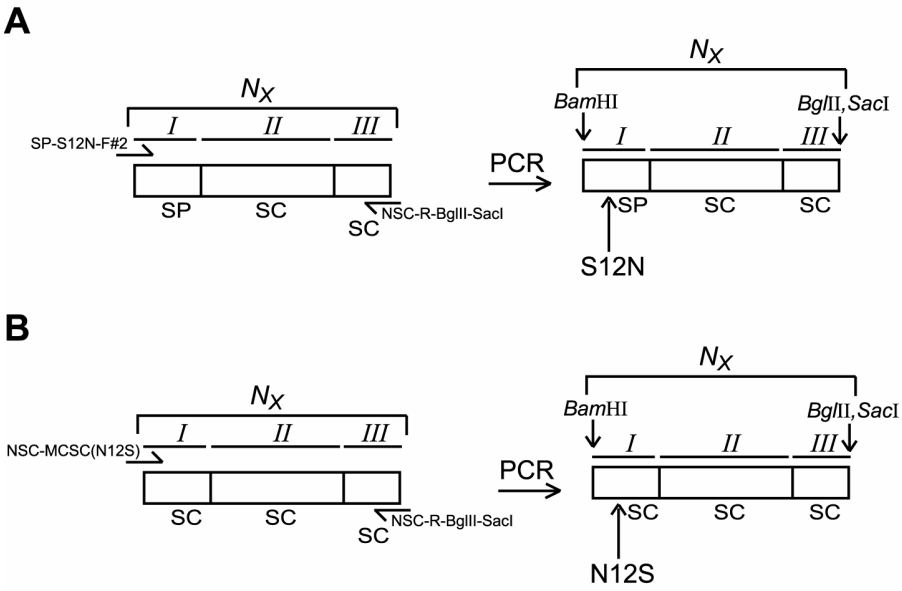


Figure 4.3 Strategy used for site-directed mutagenesis at amino acid position 12 of the prion domain
The recognition sites for restriction endonucleases *Bam*HI, *Bgl*II and *Sac*I are shown. Primers SP-S12N-F#2, NSC-R-BglII-SacI and NSC-MCSC(N12S) (Appendix C) are aligned with templates. Arrows indicate the positions of point mutations S12N and N12S, respectively. Designations are as Figure 4.2.

from p316-PS-SUP35NSC-MCSC ((1), see Materials and methods of Chapter 3 above for details). The N12S substitution was introduced through PCR-amplification by the forward primer (Figure 4.3 B). This fragment was digested with *Bam*HI and *Sac*I, and was inserted into pBSKII(+) (Stratagene) digested with the same restriction endonucleases. *SUP35N* of *S. cerevisiae* with N12S substitution was sequenced, and removed by *Bam*HI and *Bgl*II digestion. The *SUP35N* of *S. cerevisiae* with N12S substitution was fused to *SUP35M* and *SUP35C* of *S. cerevisiae*, and moved under endogenous promoter of the *S. cerevisiae SUP35* gene as described previously ((1), see Materials and methods of Chapter 3 above for details).

4.3 Results

4.3.1 Construction of plasmids with the chimeric Sup35 prion domains

By using *Hin*dIII and *Pfl*MI restriction sites, we have generated a set of *SUP35* genes containing chimeric *SUP35N* domains (see Materials and methods, and Figure 4.2 for more detail). These domains were fused in frame to the *SUP35MC* portion of *S. cerevisiae* origin and placed under the control of the endogenous *S. cerevisiae SUP35* (P_{SUP35}) promoter, located on a low-copy (centromeric) plasmid with *URA3* marker (Figure 4.4 A). For each chimeric gene with module I and modules (II-III) exchanged, an overexpressor yeast plasmid has also been generated by moving chimeric *SUP35N* domain in frame with *SUP35MC* of *S. cerevisiae* downstream of galactose-inducible (P_{GAL}) promoter (Figure 4.4 B). In our chimeric *SUP35N* constructs, we had module I of *S. cerevisiae*, *S. paradoxus* or *S. bayanus* with modules (II-III) (together) of another species (Figure 4.4 C). In addition, we have constructed chimeric *SUP35N* containing modules (I-II) from *S. cerevisiae* and module III from *S. bayanus*, as well as a construct containing module I of *S. cerevisiae* in conjunction with modules (II-III) of *S. bayanus* containing an insertion of one *S. cerevisiae* repetitive unit into the ORs region, thus restoring its length to the size equal to *S. cerevisiae* (Figure 4.4 C).

4.3.2 Prion formation by chimeric Sup35 proteins *in S. cerevisiae*

In order to test whether the Sup35 proteins with chimeric Sup35N domains remain

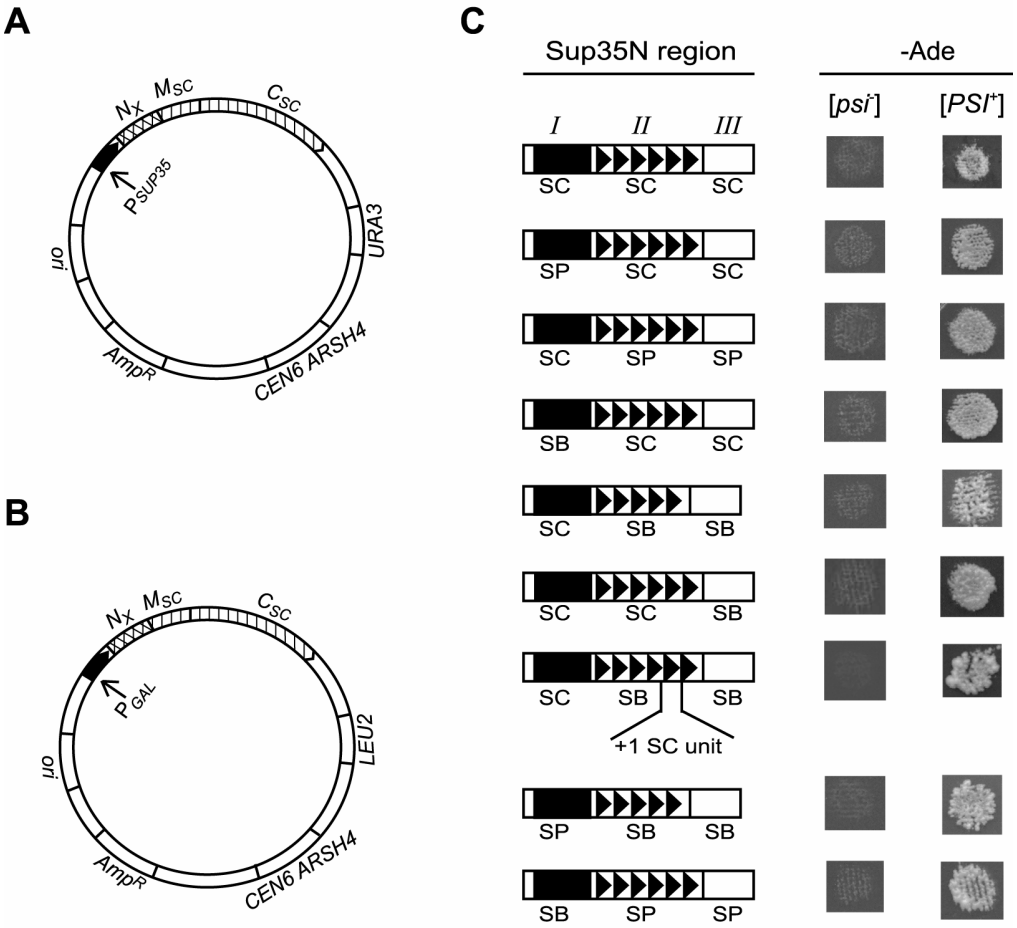


Figure 4.4 Chimeric *Saccharomyces* Sup35 proteins
(A-B) Prototype yeast shuffle plasmids used in this study. N, M and C refer to Sup35N, Sup35M and Sup35C regions, respectively. *CEN6*, *ARS H4* and *ori* refer to yeast centremere, yeast autonomously replicating sequence, and bacterial origin of replication, respectively. N_X – Sup35N region (from *S. cerevisiae*, *S. paradoxus*, *S. bayanus*, or chimeric) fused to M and C region of *S. cerevisiae* (M_{SC} and C_{SC}, respectively). P_{SUP35} - endogenous *S. cerevisiae SUP35* promoter. P_{GAL} – galactose-inducible promoter. *URA3* and *LEU2* – yeast selectable markers. Amp^R – bacterial selectable marker (ampicillin resistance). (C) Structures of chimeric Sup35N regions constructed in this work and their abilities to retain prion-forming properties. Prion presence is detected by growth on –Ade medium due to readthrough of $ade1\text{-}14_{SC}$ reporter in the [PSI^+] strain, in contrast to isogenic [psi^-] strain. Designations are the same as Figure 4.1. "+1 SC" unit indicates the *S. bayanus* region II with one extra repetitive unit of *S. cerevisiae* origin added. In each case, the Sup35N region shown on figure was fused to the Sup35M and Sup35C regions of *S. cerevisiae* origin, as indicated on panel A.

functional and retain the ability to form prions, we employed the plasmid shuffle technique described previously ((1), see above Chapter 3, Figure 3.4 A-B for details) to construct a series of the isogenic [*psi*$^-$] *S. cerevisiae* strains, each containing *sup35Δ* deletion on the chromosome and one of the *SUP35* constructs under P_{SUP35} promoter on a centromeric plasmid (Appendix A). Each strain also contained the reporter UGA allele (*ade1-14*$_{SC}$), which is suppressed due to readthrough in case when Sup35 function in termination is decreased, resulting in Ade$^+$ phenotype ((17), see above Chapter 1, Figure 1.4 for details). No growth on -Ade was detected in any [*psi*$^-$] strains containing a chimeric construct, confirming that *ade1-14*$_{SC}$ is not suppressed and therefore all constructs are fully functional in translation termination (Figure 4.4 C).

Each strain used in these experiments also contained [*PIN*$^+$], the prion form of Rnq1 protein (61, 62). It is known that transient overproduction of Sup35 or Sup35N induces *de novo* [*PSI*$^+$] formation in the strains containing [*PIN*$^+$] (17-20). Moreover, *de novo* prion induction by Sup35 overproduction may occur across the species boundaries ((1, 31), see above Chapter 3 for details), that distinguishes it from transmission of a pre-existing prion state, exhibiting a species barrier. We have transformed each of the [*psi*$^-$ *PIN*$^+$] strains, containing the chimeric constructs, with the overexpressor plasmids bearing either the same chimeric *SUP35* gene (if available) or one of the "parental" *SUP35* genes (or *SUP35N*) domains under the control of the galactose-inducible (P_{GAL}) promoter. In each strain, galactose-inducible overproduction leaded to generation of heritable Ade$^+$ derivatives (Figure 4.4 C and 4.5, Table 4.1 and Appendix E). One exception was a construct containing module I from *S. bayanus* and modules (II-III) from

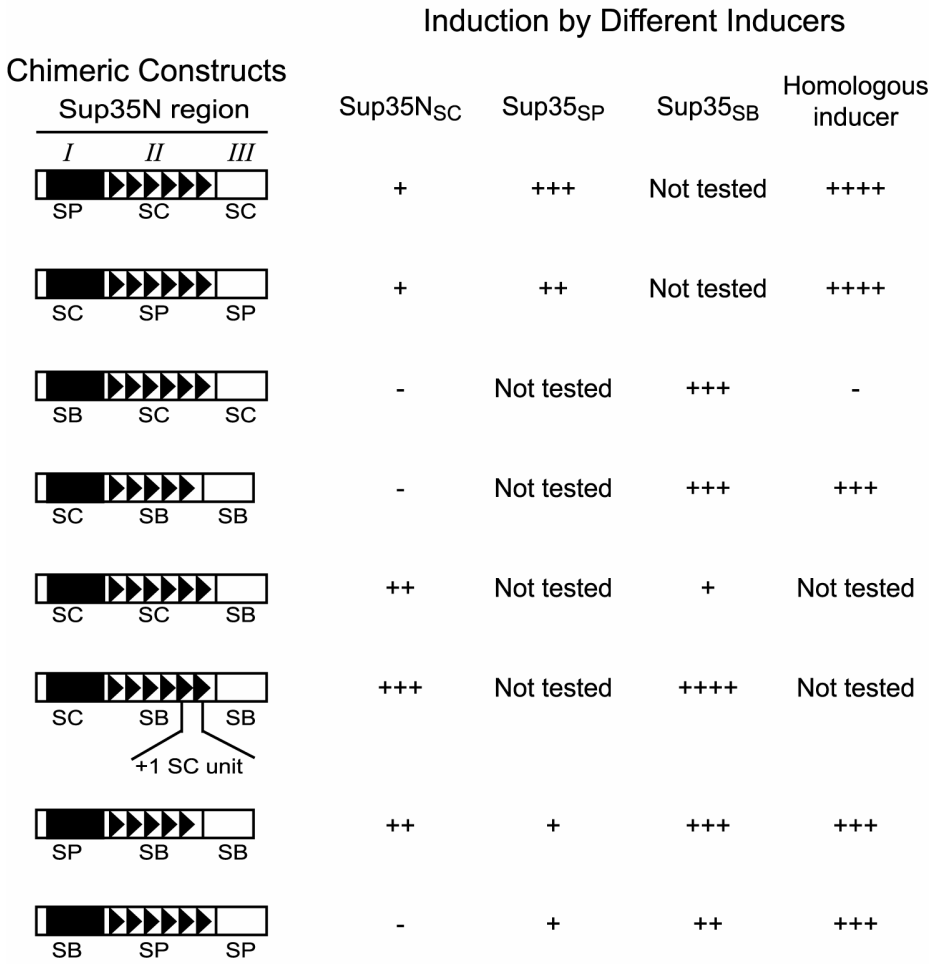


Figure 4.5 Prion induction of Sup35 proteins with chimeric prion domains
"+" and "-" refer to proof and lack of prion induction, respectively. Number of "+" refers to efficiency. Designations of the Sup35N fragments and their origins are the same as Figure 4.1 and 4.4.

S. cerevisiae, in which Ade$^+$ colonies were not induced after overproduction of the same chimeric protein, but were induced after overproduction of complete *S. bayanus* Sup35. For the construct containing modules (I-II) of *S. cerevisiae* and module III of *S. bayanus*, and the construct containing module I of *S. cerevisiae* in conjunction with modules (II-III) of *S. bayanus* containing an insertion of one *S. cerevisiae* repetitive unit into the ORs region, both parental overexpressors induced Ade$^+$ colonies although the homologous

Table 4.1 Mitotic stability of the [*PSI*$^+$] prions generated by chimeric Sup35 proteins (see Appendix E for details)

SUP35N			[*PSI*$^+$] isolates				
I	***II***	***III***	**Strong 100% stability**	**Strong 93.8-99.4% stability**	**Weak 100% stability**	**Weak 16.1-86.3% stability**	**Total number checked**
SP	SC	SC	9	6	3	0	18
SC	SP	SP	13	0	4	0	17
SB	SC	SC	6	0	0	0	6
SC	SB	SB	5	0	4	0	9
SC	SC	SB	21	1	0	0	22
SP	SB	SB	1	0	5	3	9
SB	SP	SP	21	1	0	0	22
SC	SB +1 SC unit	SB	2	0	0	8	10

In each case, [*PSI*$^+$] culture was grown on –Ura-Trp-Ade(-Ura-Leu-Ade) medium for 14 days, streaked out on YPD medium for single colonies, and from YPD to –Ade, Ade$^+$ colonies were checked for presence of [*PSI*$^+$]. All [*PSI*$^+$] isolates listed in this table were turned into [*psi*$^-$] after 3 passages on YPD medium with 5 mM GuHCl. "Strong" and "weak" refer to the ability to grow on –Ade medium.

inducers were not available (Figure 4.5). In all cases, *de novo* induced Ade$^+$ phenotype was curable by growth on the medium containing 5 mM guanidine hydrochloride (GuHCl), an agent known to counteract propagation of [*PSI*$^+$] (17). These data confirm that all chimeric Sup35 proteins retain the ability to form the [*PSI*$^+$] prion in *S. cerevisiae.*

Endogenous [*PSI*$^+$] prions of *S. cerevisiae* are divided into "strong" and "weak" "strains" or "variants", that differ from each other by both suppressor efficiency and mitotic stability. Strong [*PSI*$^+$] variants grow faster on –Ade medium and exhibit 100% stability in mitotic divisions, while weak [*PSI*$^+$] variants grow slower on –Ade medium and exhibit detectable prion loss in mitotic divisions (18). The *S. paradoxus* and *S. bayanus*

Sup35 proteins generated only mitotically unstable [*PSI*$^+$] variants in the *S. cerevisiae* cell environment, while substitution of the Sup35MC region with the respective region of *S. cerevisiae* restored the ability to produce mitotically stable prion variants by the construct derived from *S. paradoxus*, but not by the one derived from *S. bayanus* ((1), see above, Chapter 3, Table 3.2 for details). We have observed that all of the proteins with chimeric Sup35N domains were capable of generating strong and mitotically stable [*PSI*$^+$] variants. However, constructs containing modules (II-III) from *S. bayanus* produced larger proportion of [*PSI*$^+$] derivatives with weak Ade$^+$ phenotype, compared to other constructs, and some of these derivatives accumulated up to 84% of Ade$^-$ colonies after about 30 generations in non-selective conditions. The addition of one repetitive unit (OR) to a construct containing module I from *S. cerevisiae* and modules (II-III) from *S. bayanus* did not increase the stringency and stability of resulting prions (Table 4.1 and Appendix E).

4.3.3 Effects of different modules of the Sup35N on prion species barrier in the plasmid shuffle assay

In order to determine which module of Sup35N controls the species specificity of prion state transmission from *S. cerevisiae* Sup35 to the other *S. sensu stricto* Sup35 proteins, each of the chimeric *SUP35* constructs described above, as well as the positive controls of *S. cerevisiae SUP35* and negative controls of *SUP35N* from *S. paradoxus* and *S. bayanus* were transformed individually into a strong or weak [*PSI*$^+$] original strain containing *sup35Δ* deletion on the chromosome and maintaining the *S. cerevisiae SUP35*

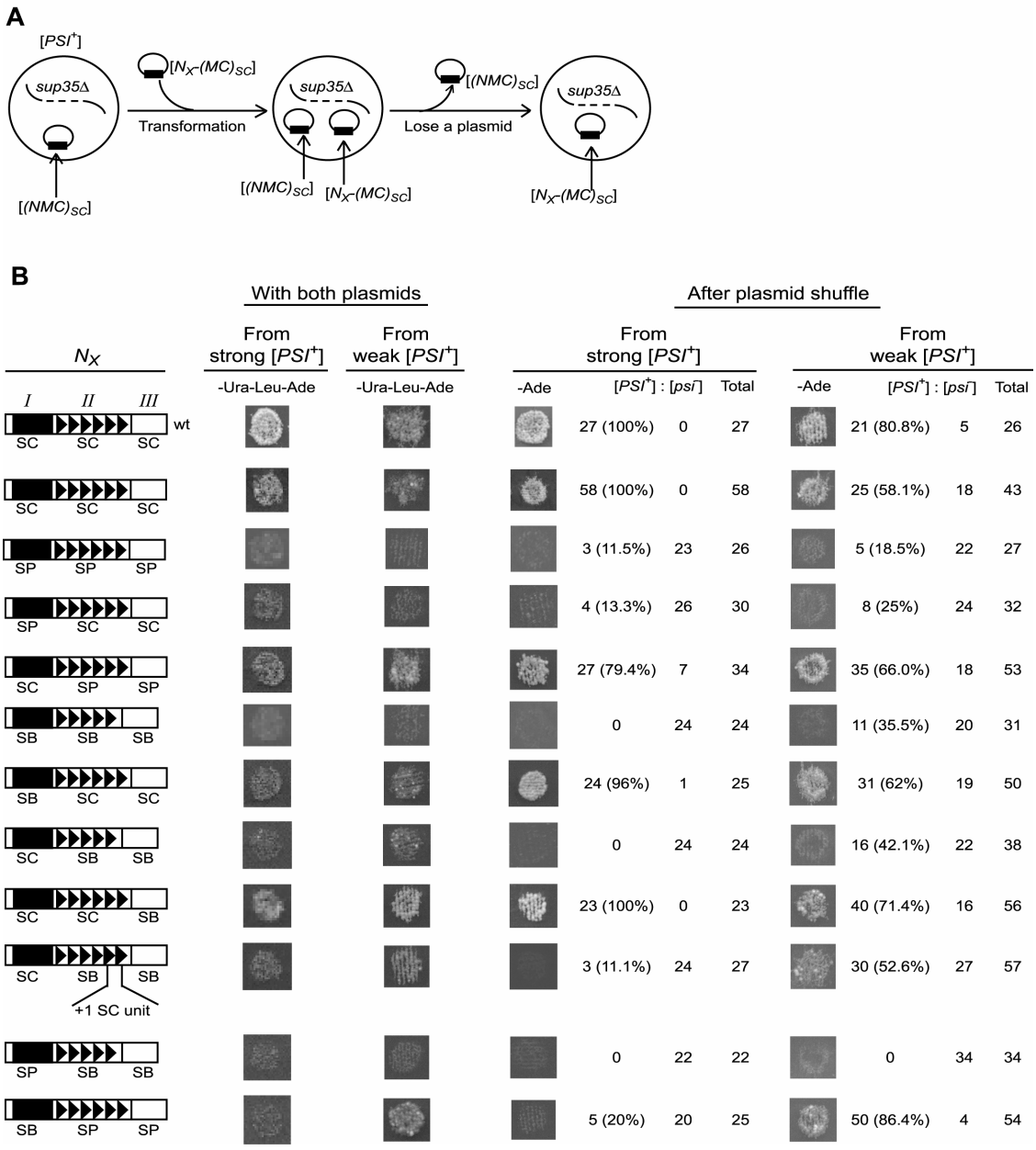


Figure 4.6 Localization of the Sup35N modules responsible for the species barrier
(A) Scheme of the plasmid shuffle experiment. Strong or weak original [*PSI*$^+$] *sup35Δ* strain with *S. cerevisiae SUP35* gene *(NMC)*$_{SC}$ on a plasmid was transformed by plasmids with chimeric prion domains (N_X), followed by loss of the original [*(NMC)*$_{SC}$] plasmid. (B) Results of the plasmid shuffle experiment, for the constructs with N_X regions as shown. Designations of the Sup35N fragments and their origins are the same as Figure 4.1 and 4.4. Results of "wt" (wild-type) from the strong [*PSI*$^+$] was presented before, and previous results of Sup35N of *S. paradoxus* and *S. bayanus* from the strong [*PSI*$^+$] were included ((1), see above Chapter 3, Figure 3.4 A-B for details). The suppression level of *ade1-14*$_{SC}$ by [*PSI*$^+$] was judged by growth on –Ura-Leu-Ade medium selective for both plasmids. Retention or loss of the prion ([*PSI*$^+$]) state was

Figure 4.6 continued
determined by growth or no growth on –Ade medium. The image of patch on –Ade medium for a representative colony from the predominant class, and numbers of [*PSI*$^{+}$] and [*psi*$^{-}$] colonies are shown in each case.

gene on a plasmid. Transformation was followed by loss of the original plasmid. As a result of this "plasmid shuffle" procedure, a plasmid with *S. cerevisiae SUP35* gene substituted by a plasmid with either a control plasmid or chimeric *SUP35* construct (Figure 4.6 A).

Introduction of a plasmid encoding Sup35 protein which does not impair the prion transmission from pre-existing [*PSI*$^{+}$], such as an additional copy of the homologous *SUP35* gene, does not affect suppression in the presence of such a plasmid, and after plasmid shuffle, a majority (or all) of colonies generated remains in prion state. However, an extra-copy of Sup35 protein which exhibits species barrier such as the construct with an heterologous *SUP35* prion domain usually decrease or eliminate suppression, and a minority (or none) of colonies generated by plasmid shuffle stays in prion state ((1), see above Chapter 3, Figure 3.4 A-B for details).

When the strong [*PSI*$^{+}$] was used as the original strain, introduction of the positive controls including wild-type *SUP35* of *S. cerevisiae*, and *SUP35N* of *S. cerevisiae* fused to *SUP35MC* of *S. cerevisiae* showed no effect on suppression, and exclusively [*PSI*$^{+}$] colonies were generated after plasmid shuffle as expected. An extra-copy of *SUP35* including *SUP35N* from *S. paradoxus* or *S. bayanus* eliminated suppression, and

predominately [*psi*$^-$] colonies were generated for *S. paradoxus*, and exclusively [*psi*$^-$] generated for *S. bayanus* after shuffle as expected (Figure 4.6 B).

An addition of chimeric *SUP35N* construct containing module I of *S. paradouxs*, and modules (II-III) of *S. cerevisiae* inhibited suppression, while the chimeric *SUP35N* construct containing module I of *S. cerevisiae*, and modules (II-III) of *S. paradoxus* had no effect. Most colonies generated by a shuffle to chimeric *SUP35N* with only module I of *S. paradoxus* and modules (II-III) of *S. cerevisiae* became [*psi*$^-$], in contrast, a majority of colonies originated from the shuffle to a chimeric construct with module I of *S. cerevisiae* and modules (II-III) of *S. paradoxus* stayed [*PSI*$^+$]. These data suggested that the module I is primarily responsible for the prion species barrier between *S. cerevisiae* and *S. paradoxus*. In *S. cerevisiae* and *S. bayanus* system, only the chimeric *SUP35N* construct containing module I of *S. cerevisiae* and modules (II-III) of *S. bayanus* inhibited suppression. In contrast, the chimeric *SUP35N* construct containing module I of *S. bayanus* and modules (II-III) of *S. cerevisiae,* and the construct containing modules (I-II) of *S. cerevisiae* and module III of *S. bayanus* did not affect suppression. The colonies generated by shuffle from *S. cerevisiae* to the construct containing module I of *S. cerevisiae*, and modules (II-III) of *S. bayanus* produced exclusively [*psi*$^-$] colonies. Shuffle from *S. cerevisiae* to the construct containing module I of *S. bayanus* and modules (II-III) of *S. cerevisiae* produced mostly [*PSI*$^+$] colonies, which demonstrated that the barrier between *S.* cerevisiae and *S. bayanus* does not depend on module I. Likewise, substitution of only module III in the *S. cerevisiae* gene with its *S. bayanus* counterpart did not impair [*PSI*$^+$] transmission, indicating that module III also plays no

role in the species barrier. Taken together, the module II is primary responsible for the barrier in this system. Remarkably, the construct containing module I of *S. cerevisiae* in conjunction with modules (II-III) of *S. bayanus* containing an insertion of one *S. cerevisiae* repetitive unit into the ORs region, which restoring its length to the size equal to *S. cerevisiae*, inhibited suppression, and transmission of the [*PSI*$^{+}$] state was not restored by insertion. Therefore, length of module II (that is, number of repeated units within the ORs region) apparently plays no role in the species barrier, and one can conclude that divergence of the module II sequences is a primary determinant of species barrier between *S. cerevisiae* and *S. bayanus*. Introduction of *SUP35N* composed with modules from *S. paradoxus* and *S. bayanus* eliminated suppression. Shuffle from *S. cerevisiae* to the construct containing module I of *S. paradoxus*, and modules (II-III) of *S. bayanus* produced exclusively [*psi*$^{-}$] colonies, and most colonies generated by a shuffle to chimeric *SUP35N* with module I of *S. bayanus* and modules (II-III) of *S. paradoxus* became [*psi*$^{-}$]. This was not surprising since the prion domain which is responsible for species barrier was of different origins, and a stronger species barrier was detected when plasmid shuffle to the chimeric *SUP35N* containing both primary responsible modules (Figure 4.6 B).

Besides using strong [*PSI*$^{+}$], the weak variant was used as the original strain. A very similar tendency was observed and generally confirmed the results obtained by using strong [*PSI*$^{+}$]. Module I is the primary responsible region between *S. cerevisiae* and *S. paradoxus*, and module II is responsible for the barrier between *S. cerevisiae* and *S. bayanus* (Figure 4.6 B).

However, there was a mild difference between the two positive controls. *SUP35N* of *S. cerevisiae* fusion to *SUP35MC* of *S. cerevisiae* created 2 aa insertion between the N and M boundary, which was not present in the wild-type Sup35 ((1), see Materials and methods of Chapter 3 above for details), and this difference decreased prion transmission, which was not observed when the strong variant was used. As all the chimeric constructs have the 2 aa insertion, the *SUP35N* of *S. cerevisiae* fusion to *SUP35MC* of *S. cerevisiae* served as the formal positive control. The difference was also shown by the construct containing module I of *S. cerevisiae* in conjunction with modules (II-III) of *S. bayanus* containing an insertion of one *S. cerevisiae* repetitive unit into the ORs region, the construct did not affect suppression, and generated as much [*PSI*$^+$] colonies as the positive control after plasmid shuffle. The additional repeat seemed to rescue the species barrier, but such effect was only shown by weak [*PSI*$^+$]. Another construct containing module I of *S. bayanus* and modules (II-III) of *S. paradoxus* showed dramatically different results. Introduction of this construct did not affect suppression, and after shuffle, it generated predominantly [*PSI*$^+$] colonies, which was probably due to the absence of the responsible modules and agreeing with the observation that these modules play no major role in the barrier in *S. cerevisiae*/*S. paradoxus* and *S. cerevisiae*/*S. bayanus* combinations, respectively (Figure 4.6 B).

The [*PSI*$^+$] colonies generated through plasmid shuffle from the original strong variant were curable by 5 mM GuHCl, an agent known to counteract propagation of [*PSI*$^+$] (17), and showed similar stringency and mitotic stability as the [*PSI*$^+$] generated by induction as described before (Table 4.2 and Appendix F).

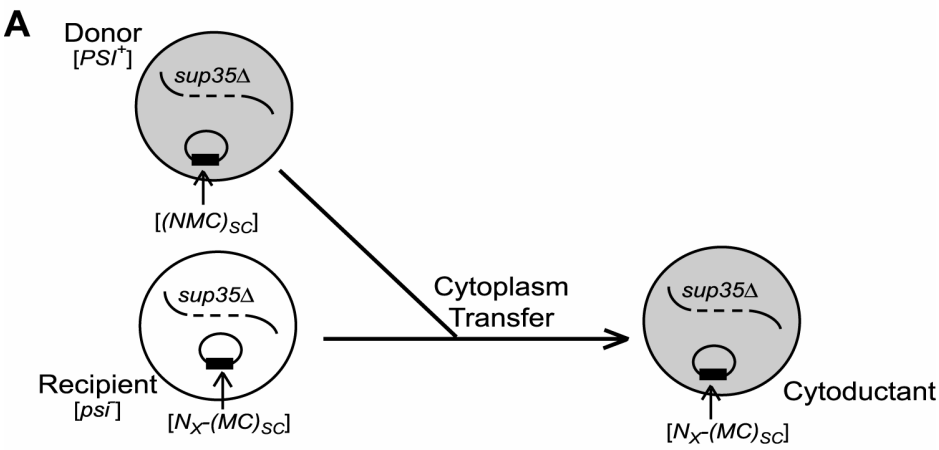
Table 4.2 Mitotic stability of the [*PSI*$^+$] prions produced by plasmid shuffle (see details in Appendix F)

SUP35N			[*PSI*$^+$] isolates				
I	***II***	***III***	**Strong 100% stability**	**Strong 90.7-99.9% stability**	**Weak 100% stability**	**Weak 71.3-99.9% stability**	**Total number checked**
SP	SC	SC	2	0	1	0	3
SC	SP	SP	5	0	7	0	12
SB	SC	SC	18	0	0	0	18
SB	SP	SP	3	0	0	0	3
SC	SC	SB	10	0	0	0	10
SC	SB +1 SC unit	SB	1	1	0	1	3

Ade$^+$ colonies produced by shuffle from *S. cerevisiae SUP35* were checked for presence of [*PSI*$^+$]. All [*PSI*$^+$] isolates listed in this table were obtained by shuffle from the strong [*PSI*$^+$] and turned into [*psi*$^-$] after 3 passages on YPD medium with 5 mM GuHCl. "Strong" and "weak" refer to the ability to grow on –Ade medium. Mosaic colonies (usually rare in strong and stable [*PSI*$^+$] isolates) were counted as [*PSI*$^+$].

4.3.4 Effects of different modules of the Sup35N on prion species barrier in the cytoduction assay

In parallel with the plasmid shuffle assay, transmission of prion state from *S. cerevisiae* to the chimeric constructs was studied by using cytoplasm transfer, or cytoduction. In these experiments, [*PSI*$^+$] strains containing the *S. cerevisiae SUP35* gene were used as cytoplasm donors. One of the donors was a strong [*PSI*$^+$] strain, while the other donor was a weak [*PSI*$^+$] strain (the same as the ones employed in the plasmid shuffle assay). In each case, donor cytoplasm was transferred to a set of the recipient karyogamy-deficient [*psi*$^-$] *sup35Δ* deletion strains bearing either control *S. cerevisiae SUP35* gene, or *SUP35*



B

N_X	Cytoductants: From strong [*PSI*+] donor			From weak [*PSI*+] donor		
I *II* *III*	[*PSI*+] : [*psi*-]		Total	[*PSI*+] : [*psi*-]		Total
SC SC SC	81 (98.8%)	1	82	78 (65.5%)	41	119
SP SP SP	69 (80.2%)	17	86	5 (8.3%)	55	60
SP SC SC	48 (81.4%)	11	59	18 (20.1%)	69	87
SC SP SP	51 (94.4%)	3	54	79(92.9%)	6	85
SB SB SB	1 (1.7%)	59	60	34 (57.6%)	25	59
SB SC SC	48 (81.4%)	11	59	56 (68.2%)	26	82
SC SB SB	0	68	68	21 (25.6%)	61	82
SC SB SB +1 SC unit	0	58	58	12 (40%)	18	30
SP SB SB	0	57	57	0	74	74
SB SP SP	5 (8.6%)	53	58	65 (75.6%)	21	86

Figure 4.7 [*PSI*+] transmission in the homologous and heterologous combinations by cytoduction

Figure 4.7 continued
(A) Scheme of the cytoplasm transfer (cytoduction) experiment. The *sup35Δ* donor strain, strong or weak [PSI^+] with *S. cerevisiae* gene on a *LEU2* plasmid, designated as $(NMC)_{SC}$ was crossed to the [psi^-] *sup35Δ* recipient strain, the *SUP35* gene containing *S. cerevisiae*, *S. paradoxus*, *S. bayanus* or chimeric *SUP35N* domain (N_X) fused to the *S. cerevisiae* M and C domain [(MC_{SC})] and located on *URA3* plasmids. Cells in which cytoplasm but not nucleus was transferred from the donor to recipient (cytoductants) were selected as described previsously ((1, 3), see above Chapter 3) (B) Results of the cytoduction experiment for the constructs with the various *SUP35N* domains are shown. Designations of the Sup35N modules and their origins are the same as in Figure 4.1 and 4.4 Transmission of the prion ([PSI^+]) state was detected by growth or no growth on -Ade medium. Numbers of independent [PSI^+] and [psi^-] cytoductants are given in each case.

genes with chimeric, *S. paradoxus* or *S. bayanus SUP35N* on a plasmid (Figure 4.7 A). For detailed description of cytoduction technique, see (1) and above Chapter 3.

The prion transmission was highly efficient from the strong [PSI^+] donor to the recipient bearing *SUP35N* of *S. cerevisiae* as expected, and weak [PSI^+] cytoduced to the same recipient strain at 66% efficiency, which indicted the control behaved very similar to the plasmid shuffle assay as described before. When the strong [PSI^+] strain with *S. cerevisiae SUP35* gene was used as a donor, prion was transmitted efficiently to the recipient strain bearing modules (II-III) of *S. paradoxus*. Prion transmission to the recipient strain bearing module I of *S. paradoxus* was decreased, but not nearly as severely as in the plasmid shuffle assay, and prion transmission to the recipient strain bearing *SUP35N* of *S. paradoxus* was further decreased a little bit, indicating that genotypic environment and/or experimental procedure may influence the stringency of the species barrier. When weak [PSI^+] strain was used as a donor, prion transmission to a recipient containing module I of *S. paradoxus* (but not to one containing modules (II-III)

of *S. paradoxus*) was dramatically decreased, and prion transmission to the recipient strain bearing *SUP35N* of *S. paradoxus* was further decreased. Taken together, these data generally confirm results of plasmid shuffle and demonstrate that module I within the prion domain is primarily responsible for species barrier between *S. cerevisiae* and *S. paradoxus* (Figure 4.7 B). They also show that species barrier between the *S. cerevisiae* and *S. paradoxus* is more pronounced in case of weak [PSI^+] strain than in case of strong [PSI^+] strain.

Cytoduction to the recipients containing the chimeric genes with *S. bayanus* modules also confirmed the plasmid shuffle data by demonstrating that *S. bayanus* module I is dispensable for the barrier, while presence of the *S. bayanus* modules (II-III) severely inhibits prion transmission. (Construct bearing only module III of *S. bayanus* was not tested in these experiments.) Interestingly, in this combination the species barrier was much more severe in case of the strong [PSI^+] donor than in case of the weak [PSI^+] donor, which is opposite to the ratio observed for *S. paradoxus*. Similar to plasmid shuffle assay, insertion of the additional repetitive unit into the *S. bayanus* OR region did not eliminate the barrier when the strong [PSI^+] was used as a donor, and the barrier was rescued in certain extend when using the weak variant (Figure 4.7 B).

Cytoduction to the recipient containing modules of *S. paradoxus* and *S. bayanus* showed very similar results to plasmid shuffle assay, too. The prion transmission to the recipient containing both responsible modules was completely eliminated regardless of the stringency of the donor strains, which agrees with the primary role of these modules in

the barrier in respective cases as described above. Very poor prion transmission to the recipient containing chimeric *SUP35N* with module I of *S. bayanus* and modules (II-III) of *S. paradoxus* was observed when the strong donor was used, which was due to a heterologous *SUP35N*, while relatively efficient prion transmission to the same recipient using weak donor was due to the absence of responsible module within the chimeric *SUP35N* (Figure 4.7 B).

4.3.5 Crucial role of the amino acid position 12 in the species barrier between *S. cerevisiae* and *S. paradoxus*.

As major determinant of the species barrier between *S. cerevisiae* and *S. paradoxus* has been confined to module I, we have focused our further attention on this region. Module I sequence exhibits higher level of divergence from *S. cerevisiae* in *S. bayanus* (5 aa substitutions out of 33 positions) than in *S. paradoxus* (3 substitutions). This seemed surprising, as module I of *S. paradoxus* is sufficient for the species barrier while module I of *S. bayanus* is not (see above, Figure 4.6 B and 4.7 B). All substitutions within module I of *S. paradoxus* are within region aa position 12 to 20, and there are only 2 substitutions within the same region of *S. bayanus*. Moreover, 2 out of 3 positions where substitutions were detected in *S. paradoxus* are also changed in *S. bayanus* sequence (Figure 4.8 A). Only change at position 12 (substitution from N to S) is specific to *S. paradoxus*. Thus, we have checked whether alteration of this specific position is responsible for the species barrier.

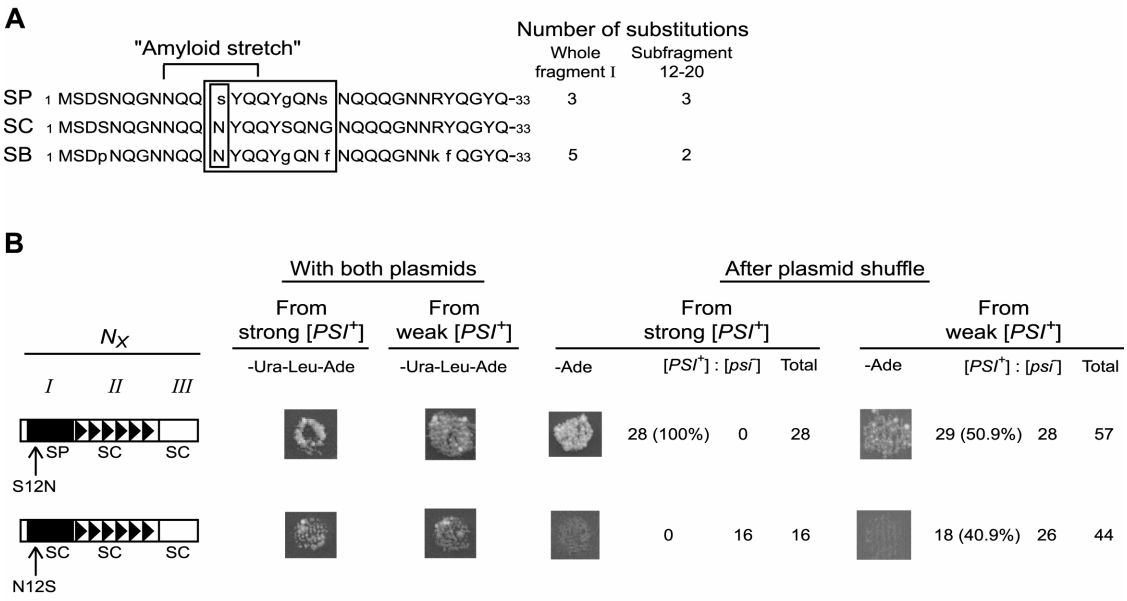


Figure 4.8 Role of amino acid residue 12 in the species barrier between *S. cerevisiae* and *S. paradoxus*
(A) Comparison of amino acid sequence of fragments I from the Sup35N regions of *S. cerevisiae*, *S. paradoxus* and *S. bayanus*. Differences from *S. cerevisiae* sequence are shown in lower case. Numbers refer to amino acid positions. Subfragment 12-20 which shows higher divergence from *S. cerevisiae* in *S. paradoxus* compared to *S. bayanus*, and position 12 where amino acid substitution is found in *S. paradoxus*, but not in *S. bayanus* are boxed. The hexapeptide sequence corresponding to "amyloid stretch" as defined by Pastor and her colleagues (2) is indicated. (B) Result of plasmid shuffle experiment. Scheme of the experiment and designations are the same as on Figure 4.6 Substitution S12N in the *S. paradoxus* sequence (indicated by arrow) eliminates the species barrier, and substitution N12S in the *S. cerevisiae* sequence (indicated by arrow) restores the species barrier.

For this purpose, we have first changed the codon for S12 (*S. paradoxus* version) into a codon for N (*S. cerevisiae* version) in the chimeric *SUP35* gene containing module I of *S. paradoxus* and modules (II-III) of *S. cerevisiae*. By using plasmid shuffle assay, we have demonstrated that S12N substitution restored suppression in the presence of such a plasmid and prion transmission from *S. cerevisiae* Sup35 protein to the mutated chimeric protein regardless of the stringency of the original strains by showing the same efficiency as controls (shown on Figure 4.6), thus eliminating the species barrier. The prions

generated through plasmid shuffle from strong variant behaved like its origin by showing strong stringency and high level of mitotic stability (Appendix F). Next, we have mutated the codon for N12 (*S. cerevisiae* version) into a codon for S (*S. paradoxus* version) in the otherwise intact *S. cerevisiae SUP35* gene. Once again, plasmid shuffle assay has demonstrated that such a single substitution inhibited suppression in the presence of such a plasmid, and blocked transmission of a prion state from *S. cerevisiae* protein to the mutant protein completely when the strong variant was used and decreased prion transmission efficiency when the weak strain was used (Figure 4.8 B). Taken together, these results show that a single aa substitution at position 12 of the Sup35 protein is both essential and sufficient for the species barrier in prion transmission from *S. cerevisiae* to *S. paradoxus*.

4.3.6 Sup35 protein with N12S mutation retains the ability of forming a prion

The single aa mutation N12S completely impaired prion transmission when the strong [PSI^+] was used. To test whether this mutation abolishes the prion-forming property, we constructed a [psi^- PIN^+] *S. cerevisiae* strain with *sup35Δ* deletion on chromosome and the *SUP35* gene with N12S mutation on a plasmid (see Materials and methods for details) by plasmid shuffle as described before in this Chapter ((1), see above Chapter 3, Figure 3.4 A-B for details). The strain contained [PIN^+], which is usually required for *de novo* [PSI^+] formation by overproduction of Sup35 (or Sup35N) (17-20). This strain was transformed with two overexpressors encoding either Sup35N of *S. cerevisiae* or module I of *S. paradoxus* and the rest part of Sup35 of *S. cerevisiae* under the P_{GAL} promoter.

Galactose-inducible overproduction leaded to strong Ade$^+$ derivates with various mitotic stability (Figure 4.9 A-B, Table 4.3). Therefore, N12S mutation did not impair the prion formation of this construct.

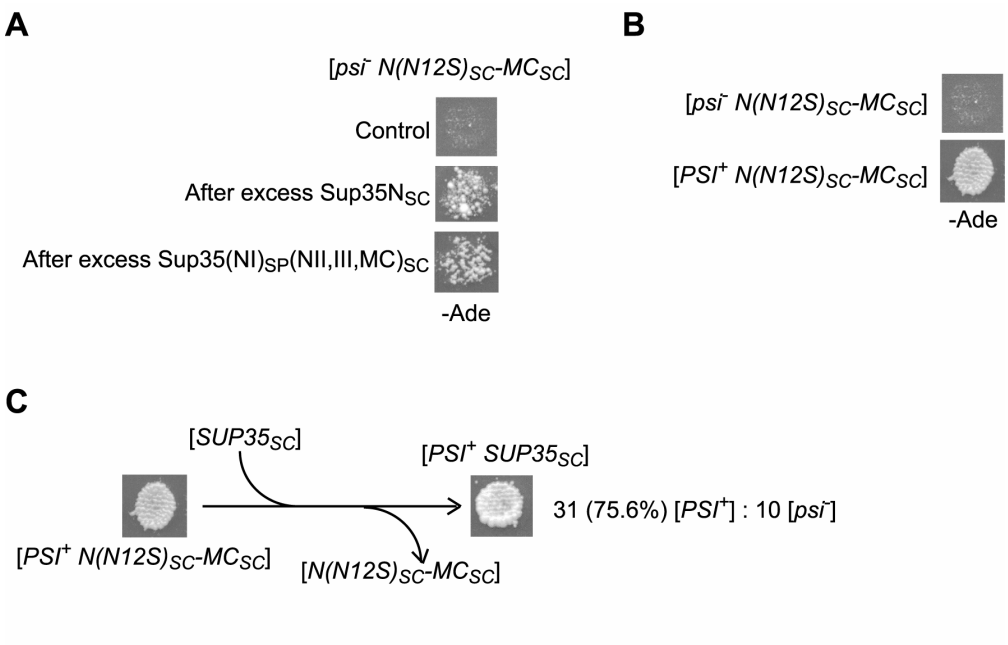


Figure 4.9 The prion-forming property of Sup35 with N12S and the prion transmission from Sup35 with N12S back to Sup35
(A) Transient overproduction of $Sup35N_{SC}$ (Sup35N of *S. cerevisiae*) or $Sup35(NI)_{SP}(NII,III,MC)_{SC}$ (only module I of *S. paradoxus* and the rest part of Sup35 of *S. cerevisiae*) induced prion formation in the [*psi*$^-$ *PIN*$^+$] *S. cerevisiae* strain bearing *N(N12S)*$_{SC}$-*MC*$_{SC}$ (*SUP35* gene with N12S mutation). Empty plasmid was used as a control. Prion formation was detected by growth on –Ade medium after galactose-inducible overproduction. (B) Sup35 protein with N12S generated strong prion variant as judged from the efficiency of *ade1-14*$_{SC}$ suppression reflected by growth on –Ade. (C) Prion transmission from Sup35 with N12S back to Sup35. The strong and mitotically stable [*PSI*$^+$] of Sup35 with N12S was transformed with *SUP35* of *S. cerevisiae* plasmid, after lost the *SUP35* of *S. cerevisiae* with N12S mutation plasmid, a majority of colonies with *SUP35* of *S. cerevisiae* plasmid remained [*PSI*$^+$], as seen by growth or no growth on –Ade medium. Numbers of [*PSI*$^+$] and [*psi*$^-$] colonies obtained are given.

Table 4.3 Mitotic stability of the Sup35 prion isolates generated by Sup35 protein with N12S mutation

Protein	Prion isolate	Number	Colonies obtained in non-selective conditions		
			[*PSI*$^+$]	[*psi*$^-$] (%)	Total
Sup35N(N12S)$_{SC}$-MC$_{SC}$	Strong	1	125	1 (0.8%)	126
		2	106	4 (3.6%)	110
		3	130	0 (0%)	130
		4	120	0 (0%)	120
		5	109	0 (0%)	109
		6	109	6(5.2%)	115
		7	107	3 (2.7%)	110
		8	86	21 (19.6%)	107

All prion isolates were induced independently of each other. All cultures were grown for at least 30 or more cell divisions in non-selective conditions. Mosaic colonies (usually rare in stable [*PSI*$^+$] isolates) were counted as [*PSI*$^+$]. "Strong" refers to intensity of growth on –Ade and color on YPD (as in Figure 4.9 B).

To test the species barrier between Sup35 of *S. cerevisiae* and Sup35 of *S. cerevisiae* with N12S in the reverse direction. The strong [*PSI*$^+$] of Sup35 with N12S generated as descried before was transformed with a plasmid of wild-type *SUP35* gene, and followed by loss of the original plasmid with N12S mutation. A majority of the colonies with the wild-type *SUP35* plasmid generated by shuffle remained in [*PSI*$^+$] state, suggesting the species barrier in the reverse direction is very weak, and the barrier exhibits asymmetric pattern.

4.4 Discussion

4.4.1 Sequence-specificity of the cross-species prion conversion

Sup35N has a QN which manifests itself as an aggregation element and promotes polymerization via intermolecular interactions, and an ORs which constitutes a propagation element. In *Saccharomyces/Candida* system, prion specificity was found to be encoded in a short region of the prion domain located at the QN, and the prion species barrier was due to the inability of heterologous prion proteins to coaggregate (21, 32-34). Another *S. cerevisiae* prion protein New1 also has Q/N-rich tracts and oligopeptide repeat regions, although their order is reversed. In Sup35/New1 system, QN-rich tract (NYN repeat), which was sufficient for aggregation, was also sufficient to specific interaction, and the mismatched sequences outside of the aggregating region did not prevent cross-interactions between heterologous proteins (15, 25, 35). In this study, we showed that different modules of the Sup35 prion domains are responsible for specificity in different combinations. In *S. cerevisiae/S. paradoxus* system, module I which contains the majority of QN is primary responsible for species-specific prion transmission, while in *S. cerevisiae/S. bayanus* system, module II which contains the very end of QN and the whole ORs is primary responsible. After carefully checking the amino acid sequence between *S. cerevisiae* and *S. bayanus*, we found that the very end of QN within module II is identical between those two species. Therefore, QN is not always the primary responsible region in controlling species barrier. What's more, the propagation element is responsible for specificity in some combination further validate our previous conclusion that the species barrier is not controlled at aggregation level ((1), see above Chapter3).

11 out of 12 single amino acid substitutions affecting Sup35 incorporation into prion aggregates *in vivo* are located between positions 8 and 26 (51), and region between amino

acids 9 and 20 is present in the amyloidogenic Sup35 peptides identified *in vitro* (63), which is agreed with that at least for the *S. cerevisiae/S. paradoxus* combination, identity of the short amino acid stretches one of which apparently including or overlapping region (9-20) appears to be more important than overall sequence divergence. This short amino acid stretch might be a region responsible for the highly specific amyloidogenic interactions both *in vivo* and *in vitro*. The species barrier between *S. cerevisiae* and *S. paradoxus* is possibly due to the impaired prion aggregation and formation, which could be detected better in case of weak [*PSI*$^+$] with bigger aggregates. The consensus six-residue "amyloid stretch" have been identified in several relevant amyloid proteins, and the *de novo* designed hexapeptide STEIIV synthesized on the bases of the sequence stretch matching the pattern has been shown to form amyloid fibirils *in vitro* (2). We found only one "amyloid stretch" within Sup35 prion domain at aa position 9 to 14, which overlaps with the region 9 to 20, and includes the amino acid position 12 which is both essential and sufficient in the *S. cerevisiae/S. paradoxus* barrier (Figure 4.8 A). In the mammalian systems, single amino acid substitutions have also been shown to block prion propagation (64, 65). The similarity between two systems indicates that the mechanism of controlling species barrier might be the same, and studying of prion transmission between closely related yeast species may provide the information useful for prediction and/or prevention of mammalian cross species prion infection.

ORs region was hypothesized to be recognized by the Hsp104 chaperone and/or its cofactors, promoting aggregate "shearing" (fragmentation), and is needed for stable inheritance of [*PSI*$^+$] aggregates (66). Weak Sup35 prions produce longer polymers,

which most likely reflects a reduced fragmentation ability (67). Sup35 of *S. bayanus* and constructs with *S. bayanus* modules (II-III) generate predominantly weak strains (see above Chapter 3 Table 3.1-2, and Chapter 4 Table 4.1 for details). In *S. bayanus*, there is one repeat missing from the ORs region ((1), Figure 4.1, see above Chapter 1, Figure 1.6 and Chpater 3, Figure 3.1 for details), therefore, one possibility could be that this emission explains predominance of weak prion and contributes the species barrier. However, addition of extra repeat doesn't increase frequency of strong prion and only slightly decreases barrier in case of strong [*PSI*$^+$] strain, indicating that sequence of ORs region is more important than number of repetitive unit. Another case when sequence variation in ORs region affected some prion patterns is the PNM2-1 strain with a point mutation within oligopeptide repeat 2 which affect prion replication (25, 66). Both cases of sequence variations in ORs region do not affect [*PSI*$^+$] aggregation and induction. The sequence differences within ORs of *S. bayanus* probably influence propagation, and PNM2-1 acts to eliminate [*PSI*$^+$] in dividing cells. The fact that ORs plays a crucial role in the barrier in the *S. cerevisiae/S. bayanus* combination, suggests that either this region is also involved in interaction specificity, or species barrier is somehow related to propagation properties. By using both plasmid shuffle assay and cytoduction assay, we have shown that species barrier in *S. cerevisiae/S. bayanus* combination is much less pronounced when the weak [*PSI*$^+$] was used as an original or donor strain, which might be because that weak strain forms longer interacting region which is more difficult for shearing (67). Longer interaction surface may make differences in short stretches less crucial.

Our data also show that module III (aa from 97 to 123 for *S. cerevisiae* and *S. paradoxus*, 90 to116 for *S. bayanus*) is not playing a significant role in the barrier. This region was implicated in binding (63), therefore, it is possible that this binding which is relatively insensitive to sequence divergence, is responsible for coaggregation between divergent proteins observed in the previous Chapter 3 (1).

4.5 Conclusions

Module I including most of the QN is responsible for the species barrier between *S. cerevisiae* and *S. paradoxus*, and module II including the very end of QN and the whole ORs regions is the primary responsible region for *S. cerevisiae* and *S. bayanus* system. As the very end of QN within the module II is identical between *S. cerevisiae* and *S. bayanus*, the ORs region is important in controlling species-specific prion transmission, and the sequence divergence within the region is more important than the number of the repeats.

A single amino acid substitution at position 12 is both essential and sufficient for prion species barrier between the Sup35 protein of *S. cerevisiae* and *S. paradoxus*.

CHAPTER 5

[*PSI*$^+$] formation in *Saccharomyces paradoxus*

5.1 Introduction

Saccharomyces paradoxus, a sister species of *S. cerevisiae*, is separated from its sister by an estimated 5 million years of evolution, and the genome was completely sequenced (39). Therefore, we took advantage of the sequenced genome, and constructed *S. paradoxus* with appropriate markers.

The amino acid sequences of N, M, and C regions of Sup35 of *S. paradoxus* show, respectively, 94%, 87%, and 100% of identity compared to *S. cerevisiae* ((1, 37-39), see above Chapter 1, Figure 1.6 for details). Sup35 of *S. paradoxus* could substitute Sup35 of *S. cerevisiae* in the *sup35Δ* deletion *S. cerevisiae* strain ((1), see above Chapter 3 for details), and formed both strong and weak [*PSI*$^+$] variants with mitotic instability in *S. cerevisiae* ((1), see above Chapter 3, Figure 3.2 and Table 3.1 for details). The *S. paradoxus* strains were used for generating [*PSI*$^+$] of Sup35 of *S. paradoxus* in the homologous cell environment.

Previously, we have demonstrated that the single amino acid at position 12 is responsible for species barrier between *S. cerevisiae* and *S. paradoxus* (see above Chapter 4, Figure 4.8 for details), and the species barrier is asymmetric between Sup35 of *S. cerevisiae* and Sup35 of *S. paradoxus* ((1), see above Chapter 3, Figure 3.5 and Table 3.4 for details).

Interestingly, the barrier between Sup35 of *S. cerevisiae* and Sup35 with N12S of *S. cerevisiae* (S12 of *S. paradoxus* version) also exhibits asymmetric pattern (see above Chapter 4, Figure 4.8 B and 4.9 C for details). However, all the *in vivo* results were obtained from experiments performed in *S. cerevisiae* cell environment. Therefore, in this study, the species barrier in *S. cerevisiae* and *S. paradoxus* system was shown by using the *S. paradoxus* strain, and species-specific prion pattern was demonstrated in parallel.

5.2 Materials and methods

5.2.1 Strains

Yeast strains used and constructed in this study are listed in Appendix A. See Results and discussion for detailed descriptions and constructions.

The *S. paradoxus* strain SP7-1D was kindly provided by G. Naumov (State Institute for Genetics and Selection of Industrial Microorganisms, Moscow, Russia). The stable *lys2* and *ura3-P2* mutants of SP7-1D have been obtained by G. Newnam in Chernoff lab using α-aminoadipic acid (α-aa) and 5-fluoroorotic acid (5-FOA) selection after 15 to 45 seconds of UV irradiation, respectively. The diploid *S. paradoxus* strain GT749-1B of the following genotype: *MATα/MATa lys2/lys2 ura3-P2/ura3-P2,* has been constructed by G. Newnam was the initial strain used for construction of *S. paradoxus* strains with appropriate markers.

5.2.2 Plasmids

HPR6.6 (kindly provided by J. Choi at School of Biology of Georgia Tech) open reading frame (ORF) was PCR-amplified from plasmid pYCL-CUP-NMScHPR6.6, which has been constructed by E. Lewitin in Chernoff lab, with restriction sites for *Sac*II and *Sac*I added upstream and downstream by primers, respectively. Plasmids pmCUP-NMSP-HPR6.6 and pmCUP-NMSB-HPR6.6 were constructed by cutting the PCR fragment with *Sac*II and *Sac*I, and inserted respectively into pmCUP-NMSPsGFP and pmCUP-NMSBsGFP ((1), see Materials and methods of Chapter 3 above for details) digested with the same restriction endonucleases to replace sGFP fragment. Plasmid pRS316-CUP-NMSC-HPR6.6 was obtained by cutting *SUP35NM* of *S. cerevisiae* fused to HPR6.6 with copper-inducible promoter upstream (P_{CUP1}-$SUP35NM_{SC}$-*HPR6.6*) from plasmid pYCL-CUP-NMScHPR6.6 with *Eco*RI and *Sac*II, and placed into pRS316GAL (52) digested with the same restriction endonucleases (See Figure 5.1 A for a prototype yeast shuffle plasmid). *ADE1* and *ade1-14* of *S. cerevisiae* ($ADE1_{SC}$ and $ade1\text{-}14_{SC}$) fragments including ORF and 500 bp upstream and 200 bp downstream were PCR-amplified from yeast genomes (*S. cerevisiae* wild-type and $ade1\text{-}14_{SC}$ strain) with restriction sites for *Pst*I and *Sac*I added upstream and downstream by primers, respectively. Plasmids pBSKII(+)-ADE1SC and pBSKII(+)-ade1-14SC were constructed by cutting PCR fragments with *Pst*I and *Sac*I, and inserting them into pBSKII(+) (Stratagene) digested with the same restriction endonucleases. Plasmids pRS317-ADE1SC and pRS317-ade1-14SC were obtained by cutting $ADE1_{SC}$ and $ade1\text{-}14_{SC}$ with 500bp upstream and 200bp downstream of ORF with *Pst*I and *Sac*I from plasmids

pBSKII(+)-ADE1SC and pBSKII(+)-ade1-14SC, and inserted into plasmid pRS317 (kindly provided by K. Lobachev at School of Biology of Georgia Tech) digested with the same restriction endonucleases, respectively (see Figure 5.1 B for a prototype yeast shuffle plasmid). All primers are listed in Appendix C.

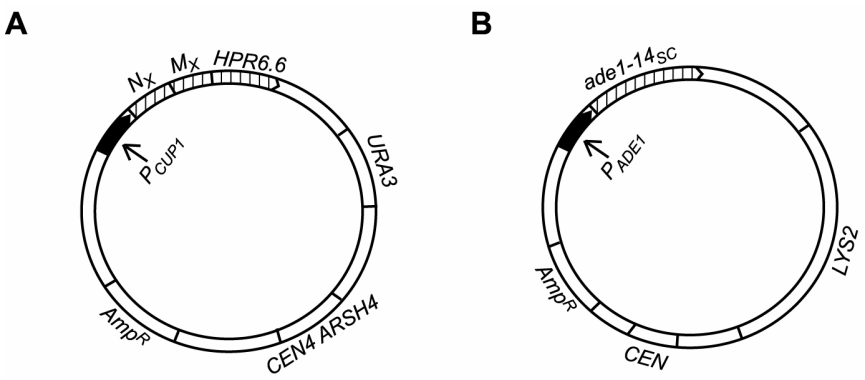


Figure 5.1 Prototype yeast shuffle plasmids used in this study
CEN and *ARS* refer to yeast centremere and yeast autonomously replicating sequence, respectively. N_X and M_X refer to Sup35N and Sup35M region (from *S. cerevisiae*, *S. paradoxus* or *S. bayanus*), respectively. P_{CUP1} – copper-inducible promoter. P_{ADE1} – endogenous *ADE1* promoter. *URA3* and *LYS2* – yeast selectable markers. Amp^R – bacterial selectable marker (ampicillin resistance).

5.2.3 Yeast growth conditions

S. paradoxus strains were grown at 25ºC. Additional 25 μM (or more) $CuSO_4$ inhibited *S. paradoxus* growth.

5.3 Results and discussion

5.3.1 Construction of haploid *S. paradoxus* strains with appropriate markers

In *S. cerevisiae*, the *HO* gene encodes an endonuclease which is involved in the cleavage of the mating type determining locus (*MAT*) to promote interconversion of mating type information in cooperation with silent mating type cassettes (*HMLα* and *HMLa*) (68). In order to make it easier to perform genetic analysis in a haploid strain displaying a stable mating type, we replaced *HO* gene by the bacterial *KANMX6* gene which confers resistance to G418/geneticin (69, 70) on the *S. paradoxus* chromosome. The diploid *S. paradoxus* strain homozygous by wild-type *HO* allele was transformed with a DNA fragment carrying *HO* gene replaced by the *KANMX6* gene. G418 resistant transformants were obtained and sporulated, and a haploid *S. paradoxus* strain with *hoΔ::KANMX6* replacement on chromosome was obtained after meiosis followed by a dissection (see construction details on Figure 5.2 A). The resulting haploid *S. paradoxus* strains could maintain stable mating type due to the lack of functional *HO* gene, and mate well with *S. cerevisiae*, which enable us to identity mating types of *S. paradoxus* haploid strains.

To check whether or not Sup35 protein can be turned into a prion state in the *S. paradoxus* cell environment, we have genetically engineered UGA reporter allele *ade1-14* of *S. cerevisiae* (*ade1-14*$_{SC}$), which is suppressed due to readthrough in case when Sup35 function in termination is decreased, resulting in Ade^{+} phenotype (17), onto *S. paradoxus* chromosome. At the first step, the *ADE1* gene of *S. paradoxus* was disrupted by *URA3* of *S. cerevisiae* (*URA3*$_{SC}$). The haploid *S. paradoxus* strain with *hoΔ::KANMX6* replacement on chromosome (obtained as described before) was transformed with a DNA fragment carrying *ADE1* gene replaced by the *URA3*$_{SC}$ gene. Ura^{+} transformants containing the *ade1Δ::URA3*$_{SC}$ transplacement on chromosome were obtained (see

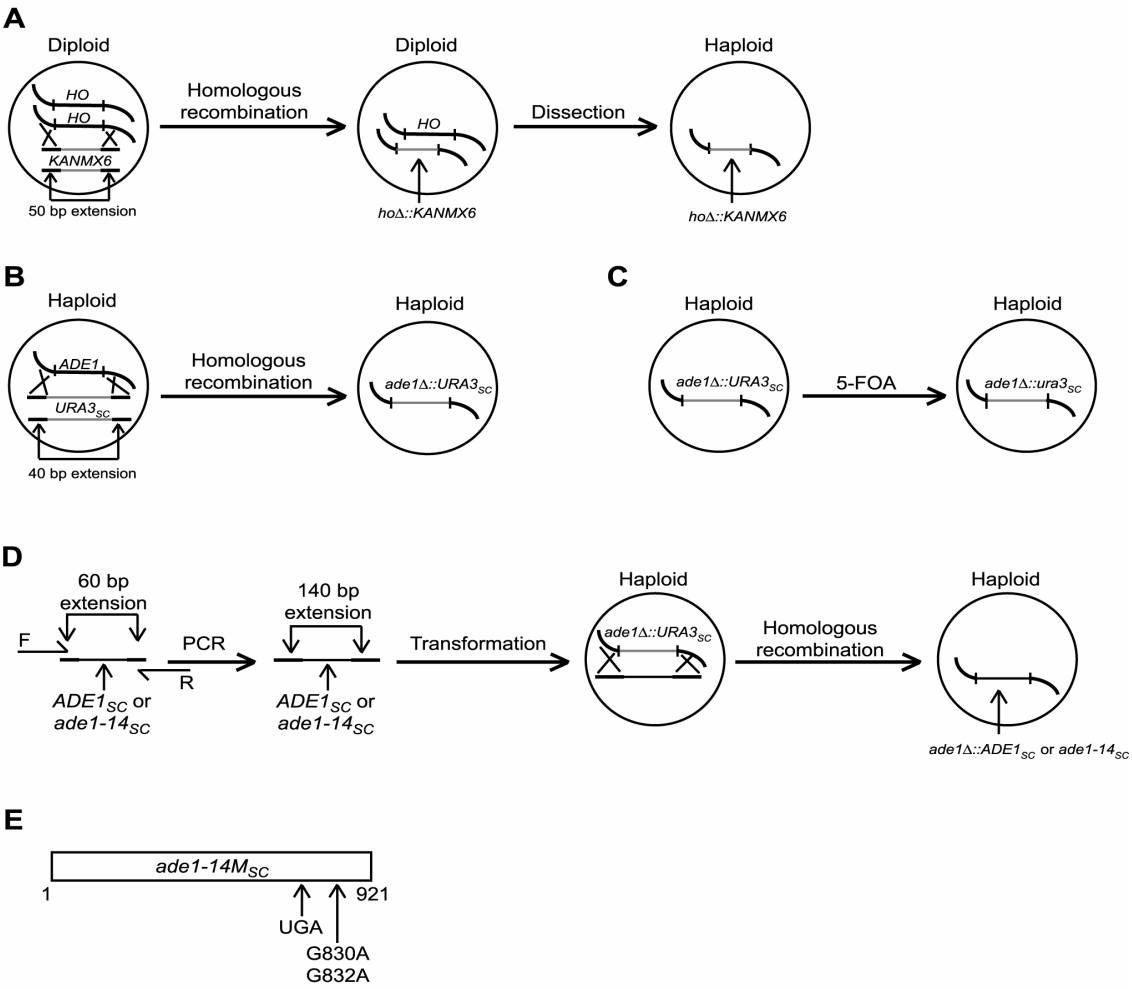


Figure 5.2 Strategy used to construct *S. paradoxus* strains
One line corresponds to the double-stranded module of DNA. (A) Replacement of *HO* by the bacterial *KANMX6* gene on the *S. paradoxus* chromosome. The *KANMX6* PCR fragment (shown in gray with less thickness) was PCR-amplified from the plasmid pFA6a-kanMX6 (71) by using primers with 50 bp 5' extensions, which were homologous to the flanking regions of the *S. paradoxus HO* gene on both sides. This fragment was transformed into a diploid *S. paradoxus* strain homozygous by wide-type *HO* allele. Resulting transformants containing a replacement of *HO* by *KANMX6* (causing resistance to G418 in yeast) generated by homologous recombination on one of the homologous chromosomes were verified by PCR. A haploid *S. paradoxus* strain with *hoΔ::KANMX6* replacement on chromosome was obtained after meiosis followed by a dissection. This strain was unable to switch mating type and diploidize due to lack of functional *HO* gene. (B) Disruption of the *ADE1* gene by *URA3* of *S. cerevisiae* (*URA3*$_{SC}$) on the *S. paradoxus* chromosome. *URA3*$_{SC}$ PCR fragment (shown in gray with less thickness) was PCR-amplified from the plasmid pBluescript-URA3 I (constructed by J. Kumar) by using primers with 40 bp 5' extensions which were homologous to the flanking regions of the *S. paradoxus ADE1* gene on both sides. This fragment was transformed into a haploid strain *S. paradoxus* (obtained as described on panel A) which contained the wild-type *ADE1*

Figure 5.2 continued
allele. Resulting Ade$^-$Ura$^+$ transformants, containing the *ade1Δ::URA3$_{SC}$* transplacement generated by homologous recombination, was verified by PCR. (C) Strategy used to construct *S. paradoxus* strain that could be transformed with the plasmids with a *URA3* marker. A haploid *ade1Δ::URA3$_{SC}$ S. paradoxus* (obtained as described on panel B) was plated onto the medium with 5-fluoroorotic acid (5-FOA), which is selective for *ura3* mutant cells. Resulting *S. paradoxus ade1Δ::ura3$_{SC}$* strain was capable of being transformed with the plasmids with a *URA3* marker. (D) Replacement of *URA3$_{SC}$* by the *S. cerevisiae* wild-type *ADE1* gene (*ADE1$_{SC}$*) or *ade1-14* (UGA) allele (*ade1-14$_{SC}$*) in a haploid *S. paradoxus ade1Δ::URA3$_{SC}$* strain. *ADE1$_{SC}$* or *ade1-14$_{SC}$* fragment (with less thickness) was PCR-amplified from yeast genome of a wild-type *ADE1* or an *ade1-14$_{SC}$ S. cerevisiae* strain by using primers with 60 bp 5' extensions, which were homologous to the flanking regions of the *URA3$_{SC}$* insertion on the *S. paradoxus* chromosome. This fragment was used as a template for the second round PCR using primers designated as F (forward) and R (reverse), and containing additional 80 bp 5' extensions homologous to the flanking *S. paradoxus* sequences located further upstream and further downstream, respectively. The purpose of this procedure was to further increase the length of the homologous regions, which would promote more efficient homologous recombination. Resulting *ADE1$_{SC}$* or *ade1-14$_{SC}$* PCR fragments with 140 bp 5' extensions on both sides were co-transformed with a *LYS2* plasmid (pRS317, kindly provided by K. Lobachev) into a haploid *ade1Δ::URA3$_{SC}$ S. paradoxus* (obtained as shown on panel B). The purpose of co-transformation was to increase the transformation efficiency. Resulting haploid strains containing *ADE1$_{SC}$* (Ade$^+$Ura$^-$) or *ade1-14$_{SC}$* (Ade$^-$Ura$^-$) alleles instead of the initial *S. paradoxus* wide-type *ADE1* allele strains were generated by homologous recombination and verified phenotypically and by PCR. In case of *ade1-14$_{SC}$* strains, several potential candidates were used in the prion induction experiments. Two candidates that were capable of prion induction (leading to Ade$^+$ phenotype) were verified by sequencing. One of them contained the *ade1-14$_{SC}$* allele with no sequence alternation, except for UGA mutation itself. Another (designated as *ade1-14M$_{SC}$*, see panel E for detail) contained two additional base substitutions apparently generated in the process of PCR, which caused amino acid substitution at positions 277 and 278. Both *ade1-14$_{SC}$* and *ade1-14M$_{SC}$* strains behaved identically in the induction and suppression experiments, indicating that additional amino acid substitutions generated in the *ade1-14M$_{SC}$* allele have no effect on the ade1 protein function. (E) Scheme of *ade1-14M$_{SC}$* allele. Numbers correspond to nucleotide positions. Premature stop codon UGA and two substitutions compared to *ADE1$_{SC}$* are shown on proportional places. Sequences are shown in Appendix G. All primers used are listed in Appendix C.

construction details on Figure 5.2 B). The haploid *ade1Δ::ura3$_{SC}$* strain of *S. paradoxus* was also obtained in parallel to allow for transformation with *URA3* plasmids (see construction details on Figure 5.2 C) In the next step, *URA3$_{SC}$* was replaced by *ADE1* of *S.*

cerevisiae (*ADE1*$_{SC}$) or *ade1-14*$_{SC}$. The haploid *S. paradoxus* strain with *ade1Δ::URA3*$_{SC}$ transplacement on chromosome (obtained as described before) was transformed with a DNA fragment carrying *URA3*$_{SC}$ gene replaced by the *ADE1*$_{SC}$ *or ade1-14*$_{SC}$ allele. Ura$^-$ transformants containing the *ade1Δ::ADE1*$_{SC}$ or *ade1-14*$_{SC}$ replacement on chromosome were obtained, and another variant, a modified *ade1-14*$_{SC}$ (*ade1-14M*$_{SC}$) with amino acid substitutions at positions 277 and 278, behaved identically as *ade1-14*$_{SC}$ in the induction and suppression experiments was obtained in parallel (see construction details on Figure 5.2 D-E, and sequences of *ade1-14M*$_{SC}$ on Appendix G). As the strain with the allele *ade1-14M*$_{SC}$ was identified first, it was used in most of the genetic experiments below.

5.3.2 Detection of prion induction of Sup35 prion in the *S. paradoxus* cell environment

Transient overproduction of Sup35 or Sup35N induces *de novo* [*PSI*$^+$] formation in the strains containing [*PIN*$^+$], the prion form of Rnq1 protein, however, the [*PIN*$^+$] requirement for [*PSI*$^+$] induction can be overcome by overproducing specific Sup35 prion-forming domain derivatives ((17-20, 61, 62, 72), and see above Chapter 1 for details). All Rnq1 protein of *S. cerevisiae* [*PIN*$^+$] strain is precipitated to the pellet at high speed, whereas all Rnq1 protein of *S. cerevisiae* [*pin*$^-$] strain retains in the soluble phase. The differential centrifugation analysis showed all Rnq1 proteins of *S. paradoxus* and *S. bayanus* remained in supernatant after centrifugation, which indicated *S. paradoxus* and *S. bayanus* are both [*pin*$^-$] strains (Figure 5.3 A). To induce a prion in the [*pin*$^-$] cell environment, we constructed a series of [*PIN*$^+$] independent inducers by fusing

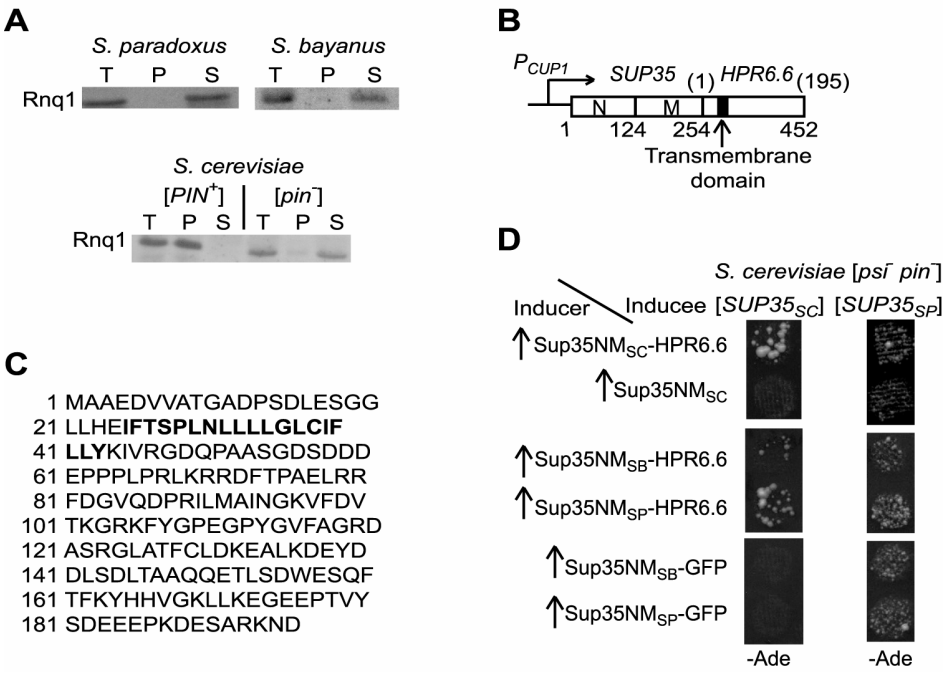


Figure 5.3 [*PSI*$^+$] prion induction in the [*PIN*$^+$] independent manner
(A) Differential centrifugation analysis of Rnq1 protein indicates that *S. paradoxus* and *S. bayanus* strains contain a non-prion form of that protein, that is, are [*pin*$^-$]. All Rnq1 protein of *S. paradoxus, S. bayanus* and [*pin*$^-$] *S. cerevisiae* is detected in supernatant (S) after centrifugation at 100,000 g, in contrast to Rnq1 protein of the control [*PIN*$^+$] *S. cerevisiae* strains detected exclusively in the pellet (P) in the same conditions. T refers to total lysate. (B) General structure of the [*PIN*$^+$] independent inducer constructs. N and M refer to *SUP35N* and *SUP35M*, respectively. Different constructs employed *SUP35NM* regions of different origins. Numbers correspond to amino acid positions, and numbers within parentheses correspond to amino acid positions of HPR6.6. Sup35NM was fused to HPR6.6, a 195 amino acid (aa) human protein with a transmembrane domain shown in black square, and placed under the copper-inducible promoter (P_{CUP1}). See Figure 5.1 A for a prototype yeast shuffle plasmid. (C) Amino acid sequence of the HPR6.6 gene. Transmembrane domain is shown in bold (4). (D) Transient overproduction of the Sup35NM-HPR6.6 fusion construct induces prion formation in the [*psi*$^-$ *pin*$^-$] strains of *S. cerevisiae*. SC, SP and SB refer to *S. cerevisiae*, *S. paradoxus* and *S. bayanus*, respectively. *S. cerevisiae* strain producing either $Sup35_{SC}$ or $Sup35_{SP}$ protein (Inducee) were transformed with the Sup35NM-HPR6.6 constructs (generated as shown on panel B), containing the Sup35NM fragments from *S. cerevisiae*, *S. paradoxus* or *S. bayanus* (as indicated). Prion formation was detected by growth on –Ade medium following transient induction on P_{CUP1} promoter on the medium with 100 μM $CuSO_4$. There was no induction observed with control plasmids expressing either $Sup35NM_{SC}$ or $Sup35NM_{SP}$ and $Sup35NM_{SB}$ fused to the Green Fluorescent Protein (GFP). Plates were photographed after 14 days of incubation.

Sup35NM of *S. cerevisiae* ($Sup35NM_{SC}$), *S. paradoxus* ($Sup35NM_{SP}$) or *S. bayanus* ($Sup35NM_{SB}$) to a human protein HPR6.6 which is also called progesterone receptor membrane component 1 (PGRMC1), and placed under copper-inducible promoter (P_{CUP1}). HPR6.6 protein contains a transmembrane domain (4), which is not present within Sup35C region (Figure 5.3 B-C, see construction details in Materials and methods and Figure 5.1 A). To verify the capability of inducing [PSI^+] in the absence of [PIN^+], the [psi^- pin^-] *S. cerevisiae* strain producing Sup35 of either *S. cerevisiae* or *S. paradoxus* on a centromeric (*CEN*) plasmid (inducee) was transformed with the [PIN^+] independent inducers (generated as described before) and controls. Transient overproduction of [PIN^+] independent inducers induced Sup35 prion in the absence of [PIN^+] in *S. cerevisiae* cell environment in most of the combinations with various efficiency. Induction by $Sup35NM_{SB}$ fused to HPR6.6 was less efficient than the other two inducers on Sup35 of *S. cerevisiae*, which is probably due to the less similarity between "inducer" and "inducee", and only the homologous inducer worked on Sup35 of *S. paradoxus*. However, transient overproduction of Sup35NM or Sup35NM-containing derivatives without HPR6.6 fusion, which is known to be able to induce [PSI^+] in the presence of [PIN^+], failed to induce Sup35 prion in the absence of [PIN^+] in *S. cerevisiae* cell environment (Figure 5.3 D). [PIN^+] was proposed to provide the initial seed to facilitate the formation of [PSI^+] (72). However, by adding the HPR6.6 tag, [PSI^+] prion could be induced in a [PIN^+]-independent manner. HPR6.6 has a highly hydrophobic transmembrane domain, which suggests intermolecular interaction of HPR6.6 may help bring Sup35NM protein together to promote aggregation.

Except for genetically engineered *ade1-14$_{SC}$* (UGA) allele on *S. paradoxus* chromosome, we also constructed *ade1-14$_{SC}$* plasmid reporter with promoter, open reading frame (ORF) and terminator regions on a plasmid (pRS317-ade1-14SC, see construction details in Materials and methods and Figure 5.1 B).

To detect prion induction in *S. paradoxus* in the presence of a plasmid-borne *ade1-14$_{SC}$* reporter, the haploid *ade1Δ::ura3$_{SC}$* (Ura$^-$) *S. paradoxus* strain (constructed as described before) was co-transformed with *ade1-14$_{SC}$* plasmid reporter and [*PIN*$^+$] independent inducers (both generated as described above in this chapter). Expression of Sup35NM$_{SP}$ fused to HPR6.6 at basal level led to efficient generation of the heritable Ade$^+$ derivatives in *S. paradoxus* (Figure 5.4 A), while the other two inducers (Sup35NM$_{SC}$-HPR6.6 and Sup35NM$_{SB}$-HPR6.6) induced heritable Ade$^+$ less efficiently (See Chapter 6, Figure 6.3 B for details). Although detailed analysis of their mitotic stability was complicated by the fact that plasmid reporter itself was unstable, frequency of the less of Ade$^+$ phenotype was increased by growth on the medium containing 5 mM guanidine hydrochloride (GuHCl), an agent known to counteract propagation of [*PSI*$^+$] (17). All the Ade$^+$ derivates obtained were mitotically unstable (data not shown).

To detect prion induction in *S. paradoxus* in the presence of chromosome *ade1-14$_{SC}$* reporter, the haploid *ade1Δ::ade1-14M$_{SC}$ S. paradoxus* strain (constructed as described before) was transformed with the plasmid encoding Sup35NM$_{SP}$ fused to HPR6.6. Transient overproduction of the inducer generated heritable Ade$^+$ derivatives that were also curable by GuHCl indicative of their prion nature, and showed various levels of

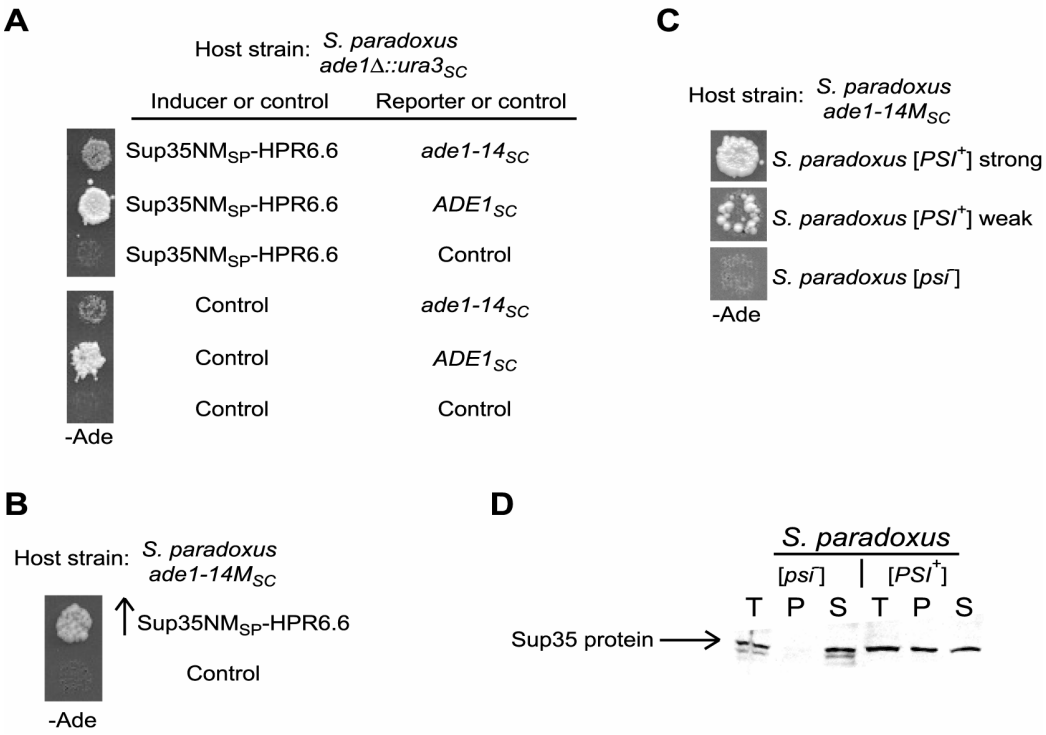


Figure 5.4 Detection of prion induction in *S. paradoxus*

SC and SP refer to *S. cerevisiae* and *S. paradoxus*, respectively. (A) Detection of prion induction in *S. paradoxus* in the strain containing plasmid *ade1-14$_{SC}$* reporter (pRS317-ade1-14SC, see Material and Methods for construction details, Figure 5.1 B for a prototype yeast shuffle plasmid). Host strain (obtained as shown on Figure 5.2 C) with *ade1Δ::ura3$_{SC}$* was co-transformed with the Sup35NM$_{SP}$-HPR6.6 based [*PIN*$^+$] independent inducer (generated as shown on Figure 5.3 B) and with *ade1-14$_{SC}$* on a *CEN* plasmid expressed from the endogenous *S. cerevisiae ADE1* promoter (Reporter). Empty vectors with the same markers but without *SUP35NM$_{SP}$-HPR6.6* or *ADE1/ade1-14$_{SC}$*, respectively, were used as negative controls. Identical *CEN* vector with wild-type *ADE1$_{SC}$* gene was used as positive control for growth on –Ade in case of *ade1-14$_{SC}$*. Prion induction was detected in case of *ade1-14$_{SC}$*(UGA) reporter as growth on -Ade medium selected for both plasmids in the presence of *P$_{CUP1}$-SUP35NM$_{SP}$-HPR6.6* construct expressed at background level (2 μM) of $CuSO_4$. Plates were photographed after 7 days of incubation. (B) Detection of prion induction in *S. paradoxus* in the presence of the chromosome *ade1-14M$_{SC}$* reporter. *S. paradoxus* strain (obtained as shown on Figure 5.2 D) with *ade1Δ::ade1-14M$_{SC}$* was transformed with the Sup35NM$_{SP}$-HPR6.6 based [*PIN*$^+$] independent inducer (generated as shown on Figure 5.3 B). Prion induction was detected in case of *ade1-14M$_{SC}$*(UGA) reporter as growth on –Ade medium after induction of Sup35NM$_{SP}$-HPR6.6 by *P$_{CUP1}$* on the medium with 10 μM $CuSO_4$. Plates were photographed after 14 days of incubation. (C) Sup35 of *S. paradoxus* generates both strong and weak prion variants in *S. paradoxus* as judged from the efficiency of *ade1-14M$_{SC}$* suppression reflected by growth on –Ade. Plates were photographed after 9 days of incubation. (D) Differentiation centrifugation analysis of strong *S. paradoxus* [*PSI*$^+$] and its isogenic [*psi*$^-$] variant indicates that while all Sup35 protein of the [*psi*$^-$] strain remains in supernatant (S) fraction after centrifugation at

Figure 5.4 continued
14,000 g, about half of Sup35 protein of the [*PSI*$^+$] is shifted to the pellet (P) strain in the same conditions. T refers to total lysate.

Table 5.1 Mitotic stability of the prion isolates generated by Sup35 protein of *S. paradoxus* in *S. paradoxus* cell environment

Prion isolate	Number	Colonies obtained in non-selective conditions		
		[*PSI*$^+$]	[*psi*$^-$] (%)	Total
Strong	1	>200	6(<3%)	>200
	2	>100	2(<2%)	>100
Weak	1	0	>100 (100%)	>100
	2	0	>100 (100%)	>100
	3	6	48(88.9%)	54
	4	0	>100 (100%)	>100
	5	0	>100 (100%)	>100
	6	0	>100 (100%)	>100
	7	7	73(91.3%)	80
	8	5	129(96.3%)	134
	9	0	>200 (100%)	>200
	10	3	81(96.4%)	84
	11	0	110(100%)	110
	12	0	>100 (100%)	>100

All prion isolates were induced independently of each other and checked for presence of [*PSI*$^+$]. All [*PSI*$^+$] isolates listed in this table were turned into [*psi*$^-$] after 3 passages on YPD medium with 5 mM GuHCl. All cultures were grown for at least 30 or more cell divisions in non-selective conditions. Mosaic colonies were counted as [*PSI*$^+$]. "Strong" and "weak" refer to intensity of growth on –Ade and color on YPD (as in Figure 5.4 C).

mitotic stability in *S. paradoxus* (Figure 5.4 B and Table 5.1). Overproduction of $Sup35NM_{SC}$-HPR6.6 induced Ade$^+$ efficiently, but $Sup35NM_{SB}$-HPR6.6 failed the induction in *S. paradoxus* probably due to a divergent Sup35NM region (data not shown).

The observation that [*PSI*$^+$] prion could be induced and propagated in *S. paradoxus* proves that prion formation by Sup35 protein is not a unique property of *S. cerevisiae*.

Formation of [URE3], the prion isoform of the nitrogen catabolism regulator Ure2 protein, was not previously detected in *S. paradoxus*, although [URE3] prion is conserved in *S. bayanus* (42). It is possible that induction of [URE3] prion in *S. paradoxus* needs a [*PIN*$^+$]-like factor or a [*PIN*$^+$]-independent inducer like the [*PSI*$^+$] induction described above.

5.3.3 Properties of Sup35 prions induced in the *S. paradoxus* cell environment

Endogenous [*PSI*$^+$] prions of *S. cerevisiae* are divided into "strong" and "weak" "strains" or "variants", that differ from each other by both suppressor efficiency and mitotic stability. Strong [*PSI*$^+$] variants grow faster on –Ade medium and exhibit 100% stability in mitotic divisions, while weak [*PSI*$^+$] variants grow slower on –Ade medium and exhibit observable prion loss in mitotic divisions (18). The same phenomenon was observed in *S. paradoxus*. Strong *S. paradoxus* [*PSI*$^+$] variant accumulated less than 3% [*psi*$^-$] colonies after 30 or more cell divisions in non-selective conditions, which is different from the strong *S. cerevisiae* [*PSI*$^+$] with 100% mitotic stability, suggesting even in the homologous cell environment, Sup35 of *S. paradoxus* could not propagate absolutely stable variant. The weak variants accumulated more than 89% [*psi*$^-$] colonies in the same conditions (Figure 5.4 C and Table 5.1). We have previously observed that Sup35 of *S. paradoxus* also forms a prion in *S. cerevisiae* cell environment, but none of them was comparable by stability to the strong [*PSI*$^+$] generated in *S. paradoxus* ((1), see above Chapter 3, Table 3.1). Therefore, prion mitotic stability is apparently controlled in part by cell cofactors (possibly chaperones) that are species-specific.

The prion isoform of Sup35 protein is insoluble and can be distinguished from the normal form of Sup35 *in vitro* by differential centrifugation analysis. Typically, the majority of Sup35 from *S. cerevisiae* [*PSI*$^+$] lysates partitions to the pellet fraction, whereas most of the Sup35 from isogenic [*psi*$^-$] lysates partitions to the soluble fraction (17). The same analysis was performed on strong *S. paradoxus* [*PSI*$^+$] and its isogenic [*psi*$^-$] strains. About half of Sup35 protein in the *S. paradoxus* [*PSI*$^+$] strain was shifted to pellet after centrifugation at 14,000 g, in contrast to the extract of its isogenic [*psi*$^-$] strain where all Sup35 protein remained in supernatant (Figure 5.4 D). This shows that Sup35 protein in the *S. paradoxus* [*PSI*$^+$] strain is in an aggregated formation similar to *S. cerevisiae* [*PSI*$^+$] strain.

5.3.4 Suppression by Sup35 prion in the *S. cerevisiae/S. paradoxus* diploid

[*PSI*$^+$] is dominant so that a *S. cerevisiae* [*PSI*$^+$] diploid strain is generated by mating a *S. cerevisiae* [*PSI*$^+$] haploid strain to a *S. cerevisiae* [*psi*$^-$] strain of the opposite mating type (17). As *S. cerevisiae* and *S. paradoxus* mate well with each other, the strong *S. paradoxus* [*PSI*$^+$] with its isogenic [*psi*$^-$] strains were mated to *S. cerevisiae* [*psi*$^-$] *sup35Δ* deletion strain with *SUP35* gene of *S. cerevisiae* or *S. paradoxus* on a plasmid of the opposite mating type. Suppression by [*PSI*$^+$] could only be detected in *S. cerevisiae/S. paradoxus* diploid homozygous by *SUP35* of *S. paradoxus*, suggesting Sup35 prion of *S. paradoxus* could be maintained in *S. cerevisiae/S. paradoxus* diploid. Heterologous Sup35 inhibited the suppression confirmed "species barrier" observed in *S. cerevisiae* cell environment ((1), see above Chapter 3, Figure 3.4 for details) (Figure 5.5 A).

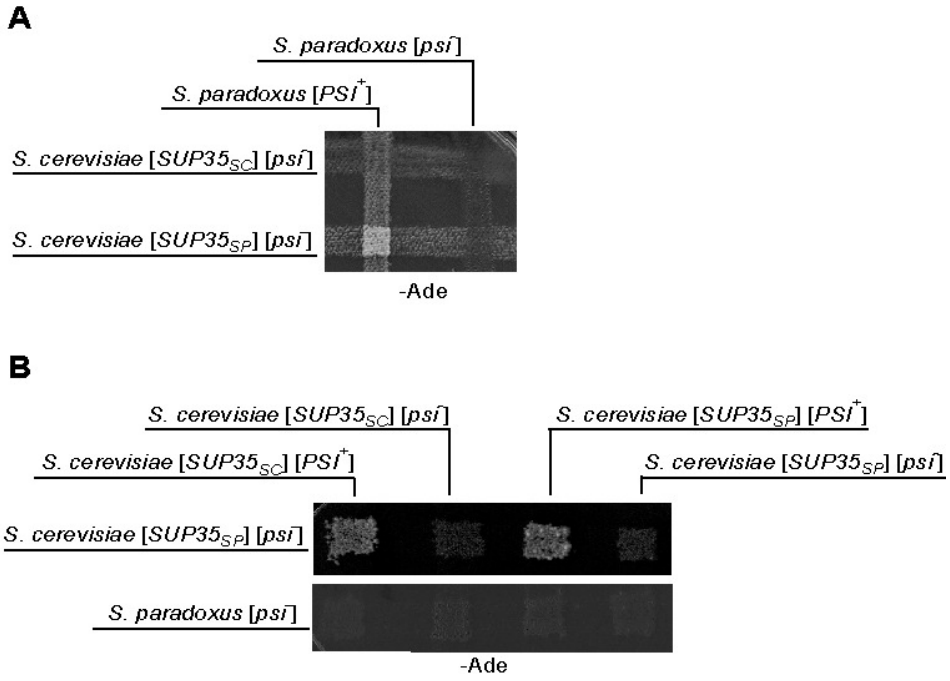


Figure 5.5 Suppression of *ade1-14$_{SC}$* (*ade1-14M$_{SC}$*) in the *S. cerevisiae/ S. paradoxus* diploid
SC and SP refer to *S. cerevisiae* and *S. paradoxus*, respectively. (A) Suppression of *ade1-14$_{SC}$* (*ade1-14M$_{SC}$*) by Sup35 prion in the *S. cerevisiae/ S. paradoxus* diploid. *S. paradoxus* strong [*PSI*$^+$] and isogenic [*psi*$^-$] strains mated to the *S. cerevisiae* [*psi*$^-$] carrying the *SUP35* gene of either *S. cerevisiae* or *S. paradoxus* as shown. Suppression by the Sup35 prion in the *S. cerevisiae/S. paradoxus* diploid homozygous by *SUP35* of *S. paradoxus* was detected by growth on –Ade medium select for plasmids. (B) Lack of suppression of *ade1-14$_{SC}$* (*ade1-14M$_{SC}$*) by Sup35 prion in the *S. cerevisiae/ S. paradoxus* diploid. *S. cerevisiae* [*PSI*$^+$] and isogenic [*psi*$^-$] carrying the *SUP35* gene of either *S. cerevisiae* or *S. paradoxus* was mated to the *S. paradoxus* or *S. cerevisiae* [*psi*$^-$] carrying the *SUP35* gene of *S. paradoxus* as shown. Suppression by the Sup35 prion in the *S. cerevisiae* diploid, but not in the *S. cerevisiae/ S. paradoxus* diploid, was detected by growth on –Ade medium select for plasmids.

The reciprocal experiment was performed by mating *S. cerevisiae* [*PSI*$^+$] and its isogenic [*psi*$^-$] *sup35Δ* deletion strain with the *SUP35* gene of either *S. cerevisiae* or *S. paradoxus* to the *S. paradoxus* [*psi*$^-$] strain of the opposite mating type. The *S. cerevisiae* [*psi*$^-$]

sup35Δ deletion strain with the *SUP35* gene of *S. paradoxus* of the same mating type as *S. paradoxus* strain was used as a control. The suppression of [*PSI*$^+$] could not be detected in *S. cerevisiae/S. paradoxus* diploid when the prion was originated from *S. cerevisiae*, while [*PSI*$^+$] dominantly inherited in the control *S. cerevisiae/S. cerevisiae* diploid (Figure 5.5 B). The lack of suppression in *S. cerevisiae/S. paradoxus* diploid homozygous by Sup35 of *S. paradoxus* when the prion was originated from *S. cerevisiae* might be due to the strength of the prion since the *S. cerevisiae* [*PSI*$^+$] strain with the *SUP35* gene of *S. paradoxus* was weak. However, loss of the weak prion apparently did not occour due to the presence of [*PSI*$^+$] in the control *S. cerevisiae/S. cerevisiae* diploid homozygous by *SUP35* of *S. paradoxus*. Another potential hypothetical explanation for lack of suppression in *S. cerevisiae/S. paradoxus* diploid in the reciprocal mating could be that the prion of the [*PSI*$^+$] *S. cerevisiae* strain with *SUP35* of *S. paradoxus* was "seeded" by *S. cerevisiae* Sup35 and remembered its origin, as the prion was generated through cross-species conversion from *S. cerevisiae* [*PSI*$^+$] with *SUP35* of *S. cerevisiae*. The *S. paradoxus* [*PSI*$^+$] is seeded by Sup35 of *S. paradoxus*. Mammalian prion also shows to remember its seeding origin (13). Heterologous Sup35 inhibited suppression as described before.

5.3.5 Prion transmission from *S. paradoxus* to *S. cerevisiae* by cytoduction

To test the efficiency of prion transmission from *S. paradoxus* to *S. cerevisiae*, we employed cytoplasm transfer (cytoduction) assay (3). For detailed description of cytoduction technique, see (1) and above Chapter 3. Cytoplasm was transferred from the

[*PSI*$^+$] *S. paradoxus* donor strain and its isogenic [*psi*$^-$] courterpart, used as a control, to two recipient karyogamy-deficient [*psi*$^-$] *sup35*Δ deletion strains, one of them containing the *SUP35* gene of *S. paradoxus*, and the other containing *SUP35* gene of *S. cerevisiae*. The [*PSI*$^+$] transmission was highly efficient in homologous combination but much less efficient in heterologous combination, which agreed with the expectation that prion transmission requires high level of homogeneity of interacting proteins, and indicate the species barrier albeit weak (Figure 5.6 A). Notably, we have not detected species barrier by this technique when prion isolate of *S. paradoxus* Sup35 generated in *S. cerevisiae* was used ((1), see above Chapter 3, Table 3.4 for details). This results suggest the stringency of the species barrier in different directions may vary, which agrees with the asymmetric barrier we observed before ((1), see above Chapter 3, Table 3.4, Figure 3.5 for details). One should note that prion isolate of *S. paradoxus* Sup35 generated in *S. cerevisiae* was seeded by *S. cerevisiae* Sup35 protein, while prion isolate generated in *S. paradoxus* was seeded by *S. paradoxus* protein. There is an alternative possibility that amyloids remember a seed, however further experiments are needed to check this.

Despite the fact that donor [*PSI*$^+$] variant of *S. paradoxus* was strong, all *S. cerevisiae* [*PSI*$^+$] cytoductants, independently of whether they contained the *SUP35* gene of *S. cerevisiae* or *S. paradoxus*, behaved as weak variants in the suppression assay. They exhibited various patterns of mitotic stability after short exposure on non-selective medium for [*PSI*$^+$], and lost prion dramatically during longer exposure on non-selective medium (YPD) (Figure 5.6 B and Table 5.2-3). While *S. cerevisiae* [*PSI*$^+$] usually keeps variant-specific patterns after cytoduction, our observation that cytoductants with *SUP35*

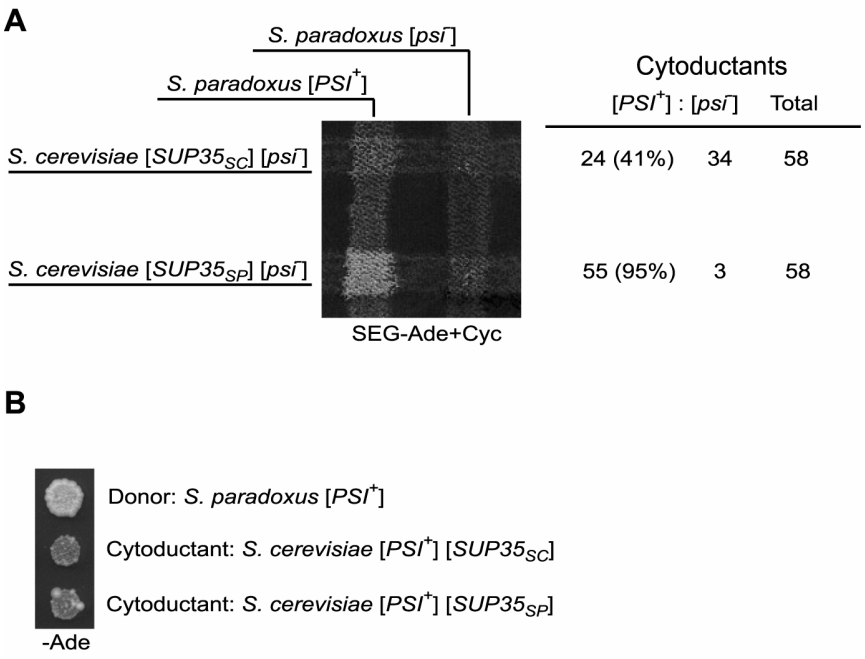


Figure 5.6 [*PSI*$^+$] transmission from *S. paradoxus* to *S. cerevisiae* in the homologous and heterologous combinations by cytoduction
SC and SP refer to *S. cerevisiae* and *S. paradoxus*, respectively. (A) The result of the cytoduction from *S. paradoxus* to *S. cerevisiae* in both homologous and heterologous combinations. The donor strains, *S. paradoxus* strong [*PSI*$^+$] and isogenic [*psi*$^-$] were crossed to the *S. cerevisiae* [*psi*$^-$] carrying the *SUP35* gene of either *S. cerevisiae* or *S. paradoxus* as shown. Cell in which cytoplasm but not nucleus was transferred from the donor to recipient (cytoductants) were selected as described previously ((3), see (1) and above Chapter 3 for detailed description of cytoduction technique). [*PSI*$^+$] transmission from *S. paradoxus* to *S. cerevisiae* was detected by growth on synthetic ethanol glycerol (SEG) medium with cycloheximide (cyc) but lacking Ade. Numbers of independent [*PSI*$^+$] and [*psi*$^-$] cytoductants are given in each case. (B) Suppression efficiency of the donor and cytoductants. The donor strains, *S. paradoxus* [*PSI*$^+$] was strong, while both cytoductants, *S. cerevisiae* [*PSI*$^+$] with *SUP35* gene of *S. cerevisiae* or *S. paradoxus*, were weak, as judged from the efficiency of *ade1-14*$_{SC}$ suppression reflected by growth on –Ade. Plates were photographed after 7 (A), and 10 (B) days of incubation.

of *S. paradoxus* gene obtained from the strong [*PSI*$^+$] *S. paradoxus* donor become weak, indicates that stringency of the prion variant could be faithfully maintained only in the homologous cell environment. Once again, this points to the existence of the species-specific cellular factors (e.g., chaperone composition) modulating the variant-specific patterns of the Sup35 prion.

Table 5.2 Mitotic stability of the prion isolates generated by cytoduction from *S. paradoxus* to *S. cerevisiae* after short exposure in non-selective conditions

Cytoductant	Prion isolate	Number	Colonies obtained in non-selective conditions		
			[*PSI*$^+$]	**[*psi*$^-$] (%)**	**Total**
[*PSI*$^+$] [*SUP35*$_{SC}$] *S. cerevisiae*	Weak	1	2	98 (98%)	100
		2	48	60 (55.6%)	108
		3	0	109 (100%)	109
		4	5	105 (95.4%)	110
		5	2	130 (98.5%)	132
		6	70	38 (35.2%)	108
		7	83	47 (36.2%)	130
		8	130	1 (0.8%)	131
		9	113	0	113
		10	104	0	104
[*PSI*$^+$] [*SUP35*$_{SP}$] *S. cerevisiae*	Weak	1	94	18 (16.1%)	112
		2	126	0	126
		3	110	7 (6.0%)	117
		4	104	0	104
		5	133	7 (5%)	140
		6	108	0	108
		7	110	0	110
		8	107	2 (1.8%)	109
		9	123	0	123
		10	104	2 (1.9%)	106

Twenty Ade$^+$ colonies produced by cytoduction from *S. paradoxus* to *S. cerevisiae* were checked for presence of [*PSI*$^+$]. All [*PSI*$^+$] isolates listed in this table were turned into [*psi*$^-$] after 3 passages on YPD medium with 5 mM GuHCl. "Weak" refers to the ability to grow on –Ade medium (Figure 5.6 B). All cultures were grown for about 5 to 10 cell divisions in non-selective conditions. Mosaic colonies were counted as [*PSI*$^+$]. Most of the [*PSI*$^+$] colonies were dark pink, and showed weak growth on –Ade.

Table 5.3 Mitotic stability of the prion isolates generated by cytoduction from *S. paradoxus* to *S. cerevisiae* after long exposure in non-selective conditions

Cytoductant	Colonies obtained in non-selective conditions		
	[*PSI*$^+$]	**[*psi*$^-$] (%)**	**Total**
[*PSI*$^+$] [*SUP35*$_{SC}$] *S. cerevisiae*	3	37 (92.5%)	40
[*PSI*$^+$] [*SUP35*$_{SP}$] *S. cerevisiae*	6	34 (85%)	40

Twenty Ade$^+$ colonies produced by cytoduction from *S. paradoxus* to *S. cerevisiae* were passed on YPD for 3 passages, and followed by streaking out for single colonies. From each original Ade$^+$ cytoductant, 4 colonies were check for [*PSI*$^+$]. Numbers correspond to the summary of 10 independent Ade$^+$ cytoductants in each case. The donor *S. paradoxus* [*PSI*$^+$] generated 100% [*PSI*$^+$] in the same condition.

5.4 Conclusions

De novo [*PSI*$^+$] induction in *S. paradoxus* indicates prion formation by Sup35 is not a unique property of *S. cerevisiae*.

Different prion variants of *S. paradoxus* Sup35 can be generated in the *S. paradoxus* cell environment, but the prion variants are not absolutely mitotically stable.

[*PSI*$^+$] could be maintained in *S. cerevisiae/S. paradoxus* diploid homozygous by *SUP35* of *S. paradoxus* when the prion is originated from a strong [*PSI*$^+$] *S. paradoxus* strain.

[*PSI*$^+$] could be transferred from *S. paradoxus* to *S. cerevisiae* by cytoduction, and the transmission is much more efficient in the homologous combination than the

heterologous combination.

Cell environment influences prion pattern, as the *S. cerevisiae* cytoductant could not reproduce the pattern of the *S. paradoxus* donor.

CHAPTER 6

Studies on [*PSI*$^+$] formation in *Saccharomyces bayanus*

6.1 Introduction

Saccharomyces bayanus, with a completely sequenced genome, is another close relative of *S. cerevisiae*, and separated from *S. cerevisiae* by an estimated 20 million years of evolution (39). N. Talarek and his colleagues have developed methods and strains for genetic studies in *S. bayanus* cell environment (44). We created *S. bayanus* strains with additional markers in this study.

The amino acid sequences of N, M and C regions of Sup35 show, respectively, 77%, 72%, and 97% of identity between *S. cerevisiae* and *S. bayanus*. *S. bayanus* ORs region is shortened by one repeat compared to *S. cerevisiae* ((1, 37-39), see above Chapter 1, Figure 1.6 and Chapter 3, Figure 3.1 for details). Sup35 of *S. bayanus* is capable of forming weak and mitotically unstable [*PSI*$^+$] in *S. cerevisiae* cell environment ((1), see above Chapter 3, Figure 3.2 and Table 3.1 for details). [URE3] is conserved in *S. bayanus* (42). What's more, we have shown that Sup35 of *S. paradoxus* could form [*PSI*$^+$] in the homologous cell environment, and proved that the prion formation of Sup35 protein is not a unique property of *S. cerevisiae* (see above Chapter 5, Figure 5.4 and Table 5.1 for details). Therefore, the same strategy was used for trying to induce [*PSI*$^+$] formation in *S.*

bayanus. However, we were unable to generate detectable prion of Sup35 in *S. bayanus* cell environment.

6.2 Materials and methods

6.2.1 Strains

Yeast strains used and constructed in this study are listed in Appendix A. See Results and discussion for detailed descriptions and constructions.

The haploid *S. bayanus* strains Su1A and Su1B (44) of genotype: *MAT***a** (or *MATα*), *ura3-1, hoΔ::KANMX4* were requested from N. Talarek, and used as the initial strains for construction of *S. bayanus* strains with appropriate markers.

6.2.2 Plasmids

Plasmids pRS41H-SUP35SC, pRS41H-SUP35SP and pRS41H-SUP35SB were constructed by cutting *SUP35* of *S. cerevisiae*, *S. paradoxus* or *S. bayanus* with *S. cerevisiae SUP35* endogenous promoter upstream (P_{SUP35}-$SUP35_{SC}$, P_{SUP35}-$SUP35_{SP}$ and P_{SUP35}-$SUP35_{SB}$) from plasmids pRS315-SUP35 (kindly provided by N. Riabinkova and S.G. Inge-Vechtomov), p315-PS-SUP35SP and p315-PS-SUP35SB ((1), see Materials and methods of Chapter 3 above for details) with *Xho*I & *Sac*I, and inserting into

pRS41H (73) digested with *Sal*I & *Sac*I, respectively (See Figure 6.1 for a prototype yeast shuffle plasmid).

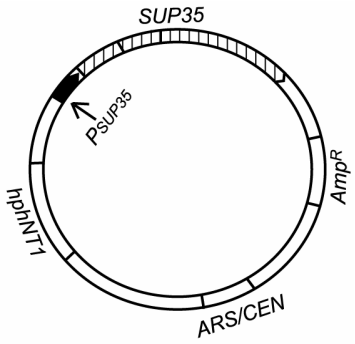


Figure 6.1 A prototype yeast shuffle plasmid used in this study
CEN and *ARS* refer to yeast centremere and yeast autonomously replicating sequence, respectively. *SUP35* gene of *S. cerevisiae, S. paradoxus* or *S. bayanus* was expressed under *S. cerevisiae SUP35* endogenous promoter (P_{SUP35}). *hphNT1* – yeast selectable marker (hygromycin B resistance). Amp^R – bacterial selectable marker (ampicillin resistance).

Plasmids pRS317-PS-SUP35SC, pRS317-PS-SUP35SP and pRS317-PS-SUP35SB were constructed by cutting P_{SUP35}-$SUP35_{SC}$, P_{SUP35}-$SUP35_{SP}$ and P_{SUP35}-$SUP35_{SB}$ from plasmids pRS315-SUP35 (kindly provided by N. Riabinkova and S.G. Inge-Vechtomov), p315-PS-SUP35SP and p315-PS-SUP35SB ((1), see Materials and methods of Chapter 3 above for details) with *Apa*I & *Sac*I, and inserting into pRS317 (kindly provided by K. Lobachev) digested with the same restriction endonucleases, respectively.

6.2.3 Yeast growth conditions

S. bayanus strains were grown at 25ºC. Additional 50 μM (or more) $CuSO_4$ inhibited *S. bayanus* growth.

6. 3 Results and discussion

6.3.1 Construction of haploid *S. bayanus* strains with appropriate markers

Sup35 protein could be turned into a prion state in *S. paradoxus* cell environment (see above Chapter 5, Figure 5.4 for details) besides in *S. cerevisiae*. To check whether or not the property of [*PSI*$^+$] formation is conserved in *S. bayanus*, we also genetically engineered UGA reporter allele *ade1-14* of *S. cerevisiae* (*ade1-14*$_{SC}$), which is suppressed due to readthrough in case when Sup35 function in termination is decreased, resulting in Ade$^+$ phenotype (17), onto the *S. bayanus* chromosome using the same strategy as before (Figure 6.2 A-B, see above Chapter 5, Figure 5.2 B, D for construction details).

To create an additional marker for further genetic analysis, a haploid *lys2 S. bayanus* strain was obtained (see construction details on Figure 6.2 C), which allow us to transform the *S. bayanus* strain with *LYS2* marker plasmids.

Sup35 proteins from *S. cerevisiae*, *S. paradoxus* and *S. bayanus* can form prions in the *S. cerevisiae* cells ((1), see above Chapter 3, Figure 3.2 C for details), and Sup35 protein from *S. paradoxus* can form prion in the *S. paradoxus* cell (see above Chapter 5, Figure 5.4 C for details). To test whether or not they are capable of generating and maintaining the prion state in the *S. bayanus* cell environment, *SUP35* genes of different origins with *S. cerevisiae SUP35* endogenous promoter (P_{SUP35}) were placed into centromeric (*CEN*)

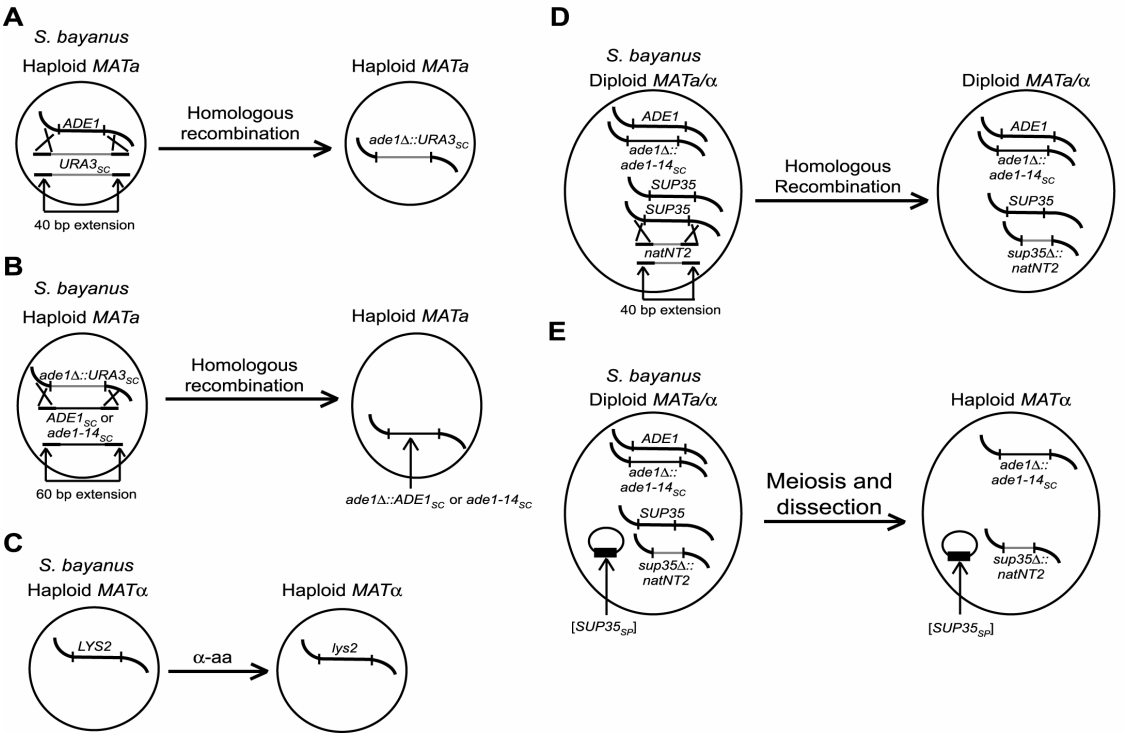


Figure 6.2 Strategy used to construct *S. bayanus* strains
One line corresponds to the double-stranded module of DNA. (A) Disruption of the *ADE1* gene by *URA3* of *S. cerevisiae* (*URA3*$_{SC}$) on the *S. bayanus* chromosome. *URA3*$_{SC}$ PCR fragment (shown in gray with less thickness) was PCR-amplified from the plasmid pBluescript-URA3 I (constructed by J. Kumar) by using primers with 40 bp 5' extensions, which were homologous to the flanking regions of the *S. bayanus ADE1* gene on both sides. This fragment was transformed into a haploid *S. bayanus* strain Su1A (44) which contained the wild-type *ADE1* allele. Resulting Ade$^-$Ura$^+$ transformants, containing the *ade1Δ::URA3*$_{SC}$ transplacement generated by homologous recombination, was verified by PCR. (B) Replacement of *URA3*$_{SC}$ by the *S. cerevisiae* wild-type *ADE1* gene (*ADE1*$_{SC}$) or *ade1-14* (UGA) allele (*ade1-14*$_{SC}$) in a haploid *S. bayanus ade1Δ::URA3*$_{SC}$ strain. *ADE1*$_{SC}$ or *ade1-14*$_{SC}$ fragment (with less thickness) was PCR-amplified from yeast genome of a wild-type or an *ade1-14*$_{SC}$ *S. cerevisiae* strain by using primers with 60 bp 5' extensions, which were homologous to the flanking regions of the *URA3*$_{SC}$ insertion on the *S. bayanus* chromosome. This fragment was transformed into a haploid *ade1Δ::URA3*$_{SC}$ *S. bayanus* (obtained as shown on panel A). Resulting haploid strains containing *ADE1*$_{SC}$ (Ade$^+$Ura$^-$) or *ade1-14*$_{SC}$ (Ade$^-$Ura$^-$) alleles instead of the initial *S. bayanus* wide-type *ADE1* allele were generated by homologous recombination and verified phenotypically and by PCR. In case of *ade1-14*$_{SC}$ strains, replacement was verified by sequencing, and contained the *ade1-14*$_{SC}$ allele with no sequence alternation, except for UGA mutation itself. (C) Strategy used to construct *S. bayanus* strain that could be transformed with plasmids with a *LYS2* marker. A haploid *S. bayanus* strain Su1B (44) was plated onto the medium with α-aminoadipic acid (α-aa), which is selective for *lys2* mutant cells. Resulting haploid *S. bayanus lys2* strain was capable of being transformed with the plasmids with a *LYS2* marker. (D) Replacement of *SUP35* by a

Figure 6.2 continued
dominant drug resistance marker, nourseothricin (*natNT2*), on *S. bayanus* chromosome. The *natNT2* PCR fragment (shown in gray with less thickness) was PCR-amplified from the plasmid pRS303N (73) with 40 bp 5' extensions, which were homologous to the flanking regions of the *S. bayanus SUP35* gene on both sides. This fragment was transformed into a diploid *S. bayanus* strain mated by a haploid *S. bayanus ade1Δ::ade1-14$_{SC}$* strain with another haploid *S. bayanus lys2* strain. The diploid *S. bayanus* was heterozygous by *ADE1/ade1Δ:: ade1-14$_{SC}$* and *LYS2/lys2*, but homozygous by wild type *SUP35* allele. Resulting transformants containing a replacement of *SUP35* by *natNT2* (causing resistance to nourseothricin) generated by homologous recombination on one of the homologous chromosomes were verified by PCR and 2:2 viability after dissection (no variable spore showed nourseothricin resistance). (E) Isolation of the haploid *S. bayanus* strains with *sup35Δ:: natNT2* replacement on chromosome. *SUP35* of *S. paradoxus* expressed from the endogenous *S. cerevisiae SUP35* promoter on a centromeric (*CEN*) plasmid with hygromycin B (*hphNT1*) resistance marker (pRS41H-SUP35SP, see Material and methods for construction details and Figure 6.1 for a prototype yeast shuffle plasmid) was transformed into a diploid *S. bayanus* strain heterozygous by *SUP35/sup35Δ::natNT2* (obtained as shown on panel D). The resulting haploid *ade1Δ:: ade1-14$_{SC}$ lys2 sup35Δ:: natNT2 S. bayanus* strain with *SUP35* of *S. paradoxus* on a plasmid was obtained after meiosis followed by a dissection, which was used as a parental strain to obtain *sup35Δ S. bayanus* strains with *SUP35* of different origins on a plasmid. All primers used are listed in Appendix C.

plasmids with either a *LYS2* or hygromycin B (*hphNT1*) marker, which could be transformed into *S. bayanus* strain (see Materials and methods for construction details and Figure 6.1 for a prototype yeast shuffle plasmid). In addition, we replaced *SUP35* gene by the gene causing a dominant resistance to the drug nourseothricin (*natNT2*) on *S. bayanus* chromosome. As Sup35 is essential for viability and translational termination (23, 24), we performed the replacement in a diploid *S. bayanus* strain. The diploid *S. bayanus* strain homozygous by wild-type *SUP35* allele was transformed with a DNA fragment carrying *SUP35* gene replaced by the *natNT2* gene. Nourseothricin resistant transformants containing the *sup35Δ::natNT2* replacement on one of the chromosomes were obtained (see construction details on Figure 6.2 D). The replacement was followed by a transformation with a plasmid containing *SUP35* of *S. paradoxus* (generated as

described before, see Material and methods for construction details and Figure 6.1 for a prototype yeast shuffle plasmid). A haploid *S. bayanus* with *sup35Δ::natNT2* replacement on chromosome and a *SUP35* plasmid was obtained after meiosis followed by a dissection (see construction details on Figure 6.2 E). As *S. bayanus* could mate with *S. cerevisiae*, we were able to identify mating types of *S. bayanus* haploid strains.

6.3.2 Attempts to induce Sup35 prion in the *S. bayanus* cell environment

As [*PIN*$^+$] independent inducers induce prion formation in both *S. cerevisiae* and *S. paradoxus* [*pin*$^-$] strains, and *S. bayanus* is a [*pin*$^-$] strain (see above Chapter 5, Figure 5.3-4 for details), the same series of inducers were used to attempt to induce Sup35 prion in the *S. bayanus* strains. The *S. bayanus* strains with chromosome *ade1-14*$_{SC}$ reporter (*ade1Δ::ade1-14*$_{SC}$) and Sup35 protein of *S. cerevisiae, S. paradoxus* or *S. bayanus* producing on a *CEN* plasmid (constructed as described before) were transformed with the [*PIN*$^+$] independent inducers, and a *S. cerevisiae* [*psi*$^-$ *pin*$^-$] strain producing Sup35 of *S. bayanus* on a *CEN* plasmid was used as a control. Transient overexpression of Sup35NM of *S. bayanus* fused to HPR6.6 induced prion in *S. cerevisiae*, but none of the [*PIN*$^+$] independent inducer induced prion in the presence of chromosome *ade1-14*$_{SC}$ reporter in *S. bayanus* (Figure 6.3 A). Using the plasmid *ade1-14*$_{SC}$ reporter, we detected prion induction in *S. paradoxus* (see above Chapter 5, Figure 5.4 A for details), and the same reporter was used for *S. bayanus* strains since there was no detectable prion induction with chromosome *ade1-14*$_{SC}$ reporter. *S. bayanus* strains producing either its own Sup35 protein on chromosome or Sup35 of *S. cerevisiae* on a *CEN* plasmid were co-

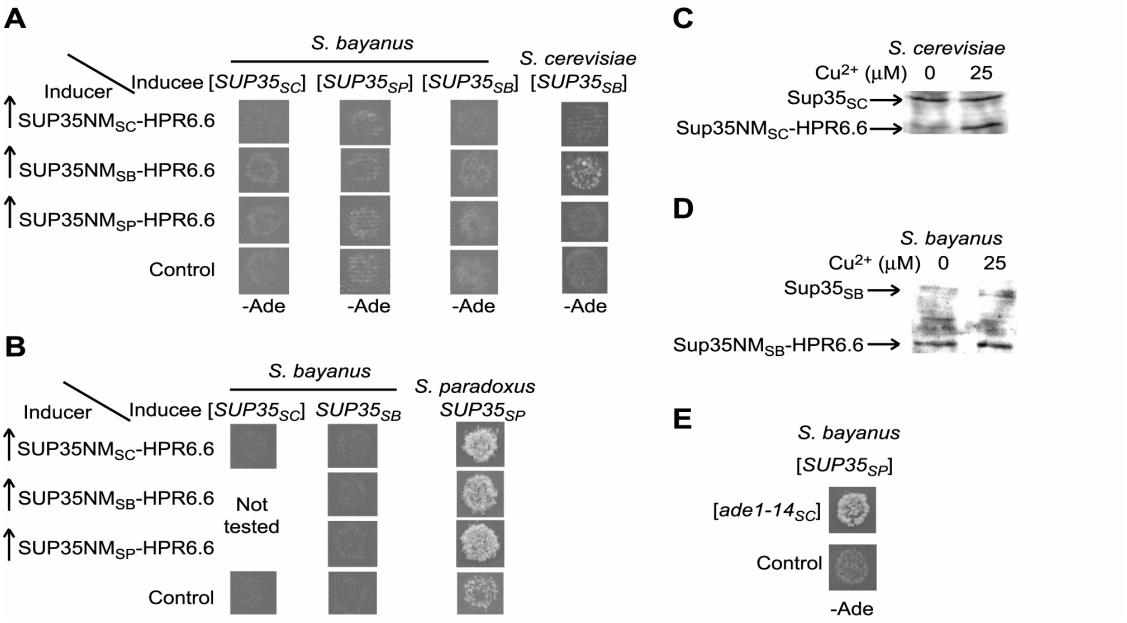


Figure 6.3 Lack of detectable prion induction in the *S. bayanus* cell environment
SC, SP and SB refer to *S. cerevisiae*, *S. paradoxus* and *S. bayanus*, respectively. (A) Summary of the Sup35 prion induction in the presence of chromosome *ade1-14$_{SC}$* reporter. *S. bayanus* strain producing Sup35$_{SC}$, Sup35$_{SP}$ or Sup35$_{SB}$ protein (Inducee) (constructed as shown on Figure 6.2 E) and *S. cerevisiae* [*psi*$^-$ *pin*$^-$] strain producing Sup35$_{SB}$ protein (Inducee) were transformed with the Sup35NM-HPR6.6 based [*PIN*$^+$] independent inducer (generated as shown on Figure 5.3 B), containing the Sup35NM fragment from *S. cerevisiae*, *S. paradoxus* or *S. bayanus* (as indicated). Prion formation was only detected in *S. cerevisiae,* but not in *S. bayanus*, by growth on –Ade medium following transient overexpression of Sup35NM$_{SB}$-HPR6.6 on P_{CUP1} promoter on the medium up to 100 μM $CuSO_4$. (B) Summary of the Sup35 prion induction in the presence of plasmid *ade1-14$_{SC}$* reporter. *S. bayanus* strain producing Sup35$_{SC}$ or Sup35$_{SB}$ protein (Inducee) (constructed as shown on Figure 6.2 E or B, respectively), and *S. paradoxus* strain producing Sup35$_{SP}$ protein (Inducee) (constructed as shown on Figure 5.2 C) were co-transformed with the Sup35NM-HPR6.6 based [*PIN*$^+$] independent inducers (generated as shown on Figure 5.3 B) and with *ade1-14$_{SC}$*(UGA) on a *CEN* plasmid (see Materials and methods of Chapter 5 and Figure 5.1 B) expressed from the endogenous *S. cerevisiae ADE1* promoter (Reporter). Prion induction was detected only in *S. paradoxus,* but not in *S. bayanus*, in case of *ade1-14$_{SC}$*(UGA) reporter as growth on -Ade medium selected for both plasmids in the presence of *P_{CUP1}-SUP35NM-HPR6.6* construct expressed at background level (2 μM) of $CuSO_4$. Plates were photographed after 7 days of incubation. (C-D) Levels of Sup35NM$_{SC}$-HPR6.6 (C) and Sup35NM$_{SB}$-HPR6.6 (D) proteins in *S. cerevisiae* and *S. bayanus*, respectively. *S. cerevisiae* (C) and *S. bayanus* (D) strains transformed with the homologous Sup35NM-HPR6.6 based [*PIN*$^+$] independent inducer (generated as shown on Figure 5.2 B) were grown in liquid media select for inducers with or without additional $CuSO_4$ added. At higher concentration (25 μM), of $CuSO_4$ did not affect levels of the full-size Sup35 proteins of *S. cerevisiae* (C) or *S. bayanus* (D) encoded by chromosomal genes, but increased level of Sup35NM$_{SC}$-HPR6.6 protein in *S. cerevisiae* dramatically (C). The level of Sup35NM$_{SB}$-HPR6.6 was quite

Figure 6.3 continued
high in *S. bayanus* at background level (2 μM) of $CuSO_4$, and was further increased at the higher concentration (25 μM) of $CuSO_4$ (D). (E) Sup35 of *S. paradoxus* could not completely substitute the function of endogenous Sup35 of *S. bayanus*. *S. bayanus* strain producing $Sup35_{SC}$ (constructed as shown on Figure 6.2 E) was transformed with *ade1-14$_{SC}$* plasmid reporter (see Materials and methods of Chapter 5 and Figure 5.1 B). Empty vector with the same marker was used as a negative control. Growth on –Ade was detected with plasmid *ade1-14$_{SC}$* reporter. Plates were photographed after 8 days of incubation.

transformed with the plasmid *ade1-14$_{SC}$* reporter and the [*PIN*$^+$] independent inducers, and the *S. paradoxus ade1Δ::ura3$_{SC}$* strain was used as a control. Transient overexpression of all [*PIN*$^+$] independent inducers induced prion in *S. paradoxus*, but none of the [*PIN*$^+$] independent inducers was able to cause detectable increase in the frequency of Ade$^+$ colonies in the presence of plasmid *ade1-14$_{SC}$* reporter in *S. bayanus* (Figure 6.3 B).The expression level of the [*PIN*$^+$] independent inducers was checked in their homologous cell environment, and Sup35 protein level was checked in parallel. Expression of Sup35 protein remained the same with or without additional $CuSO_4$ in the homologous cell environment (Figure 6.3 C-D), however, the antibodies against Sup35NM of *S. cerevisiae* could not recognize Sup35NM (or full-size Sup35) of *S. bayanus* with high efficiency, which was probably due to the protein diversity. Sup35NM of *S. cerevisiae* fused to HPR6.6 ($Sup35NM_{SC}$-HPR6.6) was induced by additional $CuSO_4$ (25 μM) in *S. cerevisiae* to such an extend comparable with Sup35 of *S. cerevisiae* ($Sup35_{SC}$) level (Figure 6.3 C), which leads to *de novo* [*PSI*$^+$] formation in *S. cerevisiae* [*pin*$^-$] strain (data not shown). The protein expression level of Sup35NM of *S. bayanus* fused to HPR6.6 ($Sup35NM_{SB}$-HPR6.6) in *S. bayanus* was much higher than Sup35 of *S. bayanus* ($Sup35_{SB}$) level at the higher concentration of $CuSO_4$ (25 μM) (Figure 6.3 D), which excluded the possibility that the lack of prion induction was due to

a low protein expression level of [*PIN*$^+$] independent inducers. Interestingly, the protein level of Sup35NM$_{SB}$-HPR6.6 in *S. bayanus* was quite high even at the background level (Figure 6.3 D), and the same situation applies to Sup35NM of *S. paradoxus* fused to HPR6.6 in *S. paradoxus* (data not shown), which leads to *de novo* [*PSI*$^+$] formation in *S. paradoxus* without additional $CuSO_4$ (Figure 6.3 B). Therefore, the protein level of the inducer is more than enough for prion formation in *S. bayanus*, however, no prion induction was detected in *S. bayanus*.

The *S. bayanus* strain with *sup35Δ::natNT2* disruption on chromosome and producing Sup35 of *S. paradoxus* on a *CEN* plasmid showed growth on –Ade with the plasmid *ade1-14$_{SC}$* reporter, indicating that Sup35 of *S. paradoxus* could not completely substitute for the lack of Sup35 of *S. bayanus* (Figure 6.3 E). The resulting Ade$^+$ phenotype was not curable by 5 mM guanidine hydrochloride (GuHCl), an agent known to counteract propagation of [*PSI*$^+$] (17), indicating non-prion nature of these Ade$^+$ colonies.

Taken together, our data show that there is no detectable prion induction in *S. bayanus* cell environment. As *ade1-14$_{SC}$* at least on the plasmid reporter is suppressible in principle in *S. bayanus*, lack of the prion detection could not be an inappropriate reporter. It is probably not due to the low expression of inducer as significant production of the chimeric protein was observed. Therefore, it appears that [*PSI*$^+$] either can not be induced in *S. bayanus* cell environment or does not cause suppression in *S. bayanus*. One possibility is that *S. bayanus* (20 million year of evolutionary distance to *S. cerevisiae*) may lose the prion formation property during the longer evolution compare to *S.*

paradoxus (5 million year of evolutionary distance to *S. cerevisiae*) which is capable of forming [*PSI*$^+$]. However, larger evolutionary distance does not simply correspond to lack of prion formation, as [URE3] is conserved in *S. bayanus* but not in *S. paradoxus* (42). Another possibility is that we need to find another [*PIN*$^+$]-independent inducer for [*PSI*$^+$] induction in *S. bayanus*.

6.3.3 Maintenance of Sup35 prion in the *S. cerevisiae/S. bayanus* diploid

[*PSI*$^+$] is dominantly inherited in the *S. cerevisiae* diploid, and [*PSI*$^+$] is maintained in *S. cerevisiae/S. paradoxus* diploid obtained by mating of a [*PSI*$^+$] haploid of *S. paradoxus* to a [*psi*$^-$] haploid of *S. cerevisiae* but not in one obtained by mating of a [*PSI*$^+$] haploid of *S. cerevisiae* to a [*psi*$^-$] haploid of *S. paradoxus* (see above Chapter 5, Figure 5.5 for details). As *S. cerevisiae* mates well with *S. bayanus*, *S. cerevisiae sup35Δ* deletion [*PSI*$^+$] and its isogenic [*psi*$^-$] strains with *SUP35* of *S. cerevisiae, S. paradoxus* or *S. bayanus* on a *CEN* plasmid were mated to *S. bayanus sup35Δ* deletion with *SUP35* of *S. cerevisiae, S. paradoxus* or *S. bayanus* on a *CEN* plasmid. [*PSI*$^+$] was maintained in all diploids having both copies of *SUP35* from the same species. In diploids homozygous by *SUP35* of *S. cerevisiae,* [*PSI*$^+$] was strong and exhibited high percentage (more than 96.7%) of mitotic stability, while in diploids homozygous by *SUP35* of *S. paradoxus* or *S. bayanus,* [*PSI*$^+$] isolates were weak (Table 6.1), as in the respective as their parental *S. cerevisiae* haploid [*PSI*$^+$] strains. Suppression by [*PSI*$^+$] was also detected in diploids bearing *SUP35* copies of different origins, but in case of when the strong *S. cerevisiae* [*PSI*$^+$] strain was used as a parent. In this case, the heterologous *SUP35* of *S. paradoxus* and *S. bayanus* partially

Table 6.1 Mitotic stability of the Sup35 prion isolates in *S. cerevisiae/S. bayanus* diploid

Protein	Prion isolate of diploids	Number	Colonies obtained in non-selective conditions		
			[*PSI*$^+$]	[*psi*$^-$] (%)	Total
$Sup35_{SC}$	Strong	1	>100	0 (0%)	>100
		2	>200	0 (0%)	>200
		3	>200	3(<1.5%)	>200
		4	>150	1 (<0.7%)	>150
		5	>150	1 (<0.7%)	>150
		6	>100	0 (0%)	>100
		7	>100	0 (0%)	>100
		8	>150	5(<3.3%)	>150
		9	>100	0 (0%)	>100
		10	>100	1(<1%)	>100
		11	>100	0 (0%)	>100
$Sup35_{SP}$	Weak	1	>100	0 (0%)	>100
		2	>100	0 (0%)	>100
		3	>100	0 (0%)	>100
		4	>100	0 (0%)	>100
		5	152	1(0.6%)	153
		6	>100	0 (0%)	>100
		7	>100	0 (0%)	>100
		8	>100	0 (0%)	>100
		9	>100	0 (0%)	>100
		10	>200	1 (<0.5%)	>200
		11	75	0 (0%)	75
$Sup35_{SB}$	Weak	1	0	>100(100%)	>100
		2	0	>100(100%)	>100
		3	104	10(8.8%)	114

Ade$^+$ diploids bearing both copies of *SUP35* from the same species were checked for presence of [*PSI*$^+$]. All cultures were grown for at least 30 or more cell divisions in non-selective conditions. Mosaic colonies were counted as [*PSI*$^+$]. "Strong" and "weak" refer to intensity of growth on –Ade and color on YPD.

inhibited suppression as described before ((1), see above Chapter 3, Figure 3.4, and Chapter 5, Figure 5.5 for details) (Figure 6.4). Lack of detectable [*PSI*$^+$] in the other heterologous *SUP35* diploid combinations was possibly due to a juxtaposition of two effects, weak suppression of the respective prion isolates and inhibition of suppression by

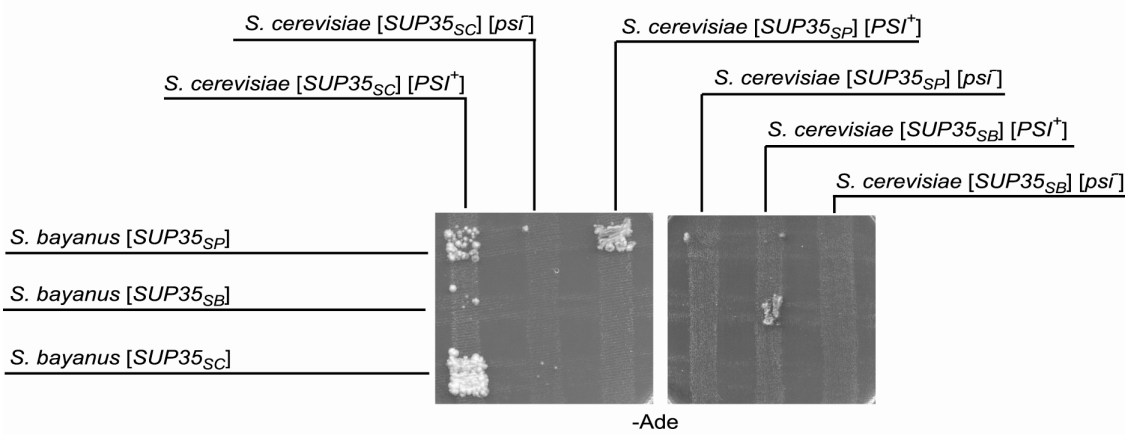


Figure 6.4 Suppression of *ade1-14$_{SC}$* in the *S. cerevisiae/S. bayanus* diploid strains obtained from [*PSI*$^+$] and [*psi*$^-$] haploids
S. cerevisiae [*PSI*$^+$] and its isogenic [*psi*$^-$] derivates carrying *SUP35* gene of different origins were mated to *S. bayanus* strains carrying *SUP35* gene of different origins as shown. Suppression of *ade1-14$_{SC}$* by Sup35 prion in the *S. cerevisiae/S. bayanus* diploid was detected by growth on –Ade selected for plasmids in all diploids homozygous by *SUP35*, and the *SUP35* heterozygous diploids mated by the strong *S. cerevisiae* [*PSI*$^+$] carrying *SUP35* gene of *S. cerevisiae* with *S. bayanus* strain carrying *SUP35* gene of *S. paradoxus* or *S. bayanus*.

a heterologous *SUP35* gene due to a species barrier. Non-selective mediums for *SUP35* plasmids (one plasmid from *S. cerevisiae* parental strains, and the other one of a different marker from *S. bayanus* parental strains) were used for checking suppression after one of the plasmids was lost. The selection for the plasmids coming from *S. bayanus* strains was apparently necessary, otherwise, the suppression by [*PSI*$^+$] haploid *S. cerevisiae* was shown. The selection for the *SUP35* of *S. paradoxus* plasmid from *S. cerevisiae* strain was also necessary since both copies of *SUP35* of *S. paradoxus* were required for termination function in *S. cerevisiae/S. bayanus* diploids. [*PSI*$^+$] was maintained in the diploid homozygous by *SUP35* of *S. cerevisiae* after losing the plasmid from the *S. cerevisiae* strain as expected. However, [*PSI*$^+$] could not be maintained in the diploid homozygous by *SUP35* of *S. bayanus* after losing the plasmid from the *S. cerevisiae*

strain, which was possibly due to loss of the weak prion in parallel with the plasmid. Lack of prion maintenance in the other heterologous diploids was due to a juxtaposition of two effects, species barrier and weak prion loss (data not shown).

[*PSI*$^+$] prion, provide by *S. cerevisiae* parental strain, was maintained in *S. cerevisiae/S. bayanus* regardless of the stringency of the original prion. However, there is no detectable suppression in *S. cerevisiae/S. paradoxus* diploid if the prion origin is provide by a weak *S. cerevisiae* [*PSI*$^+$]. The difference suggests the role of cell environment controlling the [*PSI*$^+$] prion propagation.

To test whether the prion can be transmitted from *S. cerevisiae* to *S. bayanus*, we employed cytoplasm transfer (cytoduction) assay ((3), for detailed description of cytoduction technique, see (1) and above Chapter 3). Karyogamy-deficient [*PSI*$^+$] *S. cerevisiae* with its isogenic [*psi*$^-$] strains were used as donor strains, and *S. bayanus* [*rho*$^-$] [*psi*$^-$] *sup35*Δ strains with *SUP35* gene of different origins were used as recipient strains. However, we could not detect transfer of cytoplasm from *S. cerevisiae* to *S. bayanus* (date not shown), indicating that cytoduction technique is inefficient when more divergent partner strains are used.

6.4 Conclusions

There is no detectable Sup35 prion formation in *S. bayanus*, and lack of prion formation was not due to a non-suppressible reporter, nor a low level of inducer proteins.

Prion could be maintained in *S. cerevisiae/S. bayanus* diploid, and the prion is originated from [*PSI*$^{+}$] *S. cerevisiae* strains regardless of the prion stringency.

OVERALL CONCLUSIONS

We have demonstrated that the closely related Sup35 proteins from the *Saccharomyces sensu stricto* group exhibit species barrier, and the barrier in the forward direction (from *S. cerevisiae* to *S. paradoxus* or *S. bayanus*) and the reverse direction (from *S. paradoxus* or *S. bayanus* to *S. cerevisiae*) was asymmetric. QN was responsible for the species barrier between *S. cerevisiae* and *S. paradoxus*, and the amino acid at position 12 (N for *S. cerevisiae*, and S for *S. paradoxus*) played crucial role of controlling species-specific prion transmission. ORs was the primary responsible region for the barrier between *S. cerevisiae* and *S. bayanus*, and the sequence divergence of this region was more important for the species barrier than the number of the ORs. These data suggests species barrier is controlled by different short amyloidogenic stretches in different combinations.

Protein aggregates of Sup35 from the *Saccharomyces sensu stricto* group co-aggregated in *S. cerevisiae* cell environment. Amyloid seeds of Sup35 proteins either promoted or delayed protein polymerization of heterologous Sup35. These data suggests closely related Sup35 proteins involve direct interactions, and the species barrier between is not controlled at protein aggregation level, but rather the conformational switch step.

Sup35 of *S. paradoxus* formed [*PSI*$^+$] in *S. cerevisiae*. Sup35 of *S. paradoxus* was also capable of forming [*PSI*$^+$] in the homologous cell environment, and the *S. paradoxus* [*PSI*$^+$] could be transmitted into *S. cerevisiae* although with varied prion pattern after transmission. However, Sup35 of *S. bayanus* could not form [*PSI*$^+$] in the homologous

cell environment despite the fact the protein was capable of forming [*PSI*$^{+}$] in *S. cerevisiae*. These data suggests [*PSI*$^{+}$] formation is not a unique property of *S. cerevisiae*, and the overall cell environment also plays role in controlling the prion pattern.

APPENDIX A.

Genotypes and origins of the yeast strains

Table A.1 *S. cerevisiae* strains used as the original source for this work

Strain	Genotype/description	Reference/ Source
GT81-1C	*S. cerevisiae MATa ade1-14$_{SC}$ his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 [PSI$^+$][PIN$^+$]*	(74)
GT159	*S. cerevisiae MATa ade1-14$_{SC}$ his3-Δ200 lys2 leu2-3,112 trp1-Δ ura3-52 [psi$^-$][PIN$^+$]*	
GT255-2A	*S. cerevisiae MATα ade1-14 $_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3 [psi$^-$][PIN$^+$] [CEN LEU2 SUP35$_{SC}$]*	(1)
GT255-2D	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3 [psi$^-$][PIN$^+$] [CEN LEU2 SUP35$_{SC}$]*	(43)
GT256-23C	*S. cerevisiae MATα ade1-1$_{SC}$ 4 his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3 [PSI$^+$][CEN LEU2 SUP35$_{SC}$]*	(1)
OT49 (S288C)	*S. cerevisiae*	ATCC
OT56	*S. cerevisiae MATa ade1-14$_{SC}$ his3- Δ200 leu2-3,112 trp1-289$_{UAG}$ ura3-52 [PSI$^+$][PIN$^+$]*	(18)

Table A.2 *S. cerevisiae* strains constructed in this work

Strain	Genotype/description	Reference/ Source	Genotypic background
GT795	*S. cerevisiae MATα ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3 [psi$^-$][PIN$^+$] [CEN URA3 SUP35$_{SP}$]*	This study	GT81(31)
GT809	*S. cerevisiae MATα ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3 [CEN URA3 SUP35$_{SC}$] [PSI$^+$]*	(1)	
GT810	*S. cerevisiae MATα ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3 [psi$^-$][PIN$^+$] [CEN URA3 SUP35$_{SC}$]*	This study	
GT811	*S. cerevisiae MATα ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3 [psi$^-$][pin$^-$] [CEN URA3 SUP35$_{SC}$]*		
GT825	*S. cerevisiae MATα ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3 [psi$^-$][PIN$^+$][CEN LEU2 SUP$_{SB}$]*	(1)	
GT870	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3 [psi$^-$][PIN$^+$] [CEN URA3 SUP35$_{SP}$]*	This study	
GT871	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3 [psi$^-$][PIN$^+$][CEN LEU2 SUP$_{SP}$]*	(1)	

Table A.2 continued

GT920	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3* [*psi$^-$*][*PIN$^+$*] [*CEN URA3 SUP35$_{SC}$*]	This study	GT81(31)
GT921	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3* [*psi$^-$*][*PIN$^+$*] [*CEN URA3 SUP35$_{SB}$*]		
GT948	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3* weak [*PSI$^+$*][*PIN$^+$*] [*CEN URA3 SUP35$_{SB}$*]		
GT953	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ leu2-3,112 trp1-289 ura3 kar1 cyh^R sup35Δ::HIS3* [*rho$^-$*] [*psi$^-$*] [*PIN$^+$*] [*CEN LEU2 SUP35$_{SC}$*]	(1)	1B-D910
GT954	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ leu2-3,112 trp1-289 ura3 kar1 cyh^R sup35Δ::HIS3* [*rho$^-$*] [*psi$^-$*] [*PIN$^+$*][*CEN LEU2 SUP35$_{SP}$*]		
GT955	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ leu2-3,112 trp1-289 ura3 kar1 cyh^R sup35Δ::HIS3* [*rho$^-$*] [*psi$^-$*] [*PIN$^+$*][*CEN LEU2 SUP35$_{SB}$*]		
GT988-1A	*S. cerevisiae MATα ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3* weak [*PSI$^+$*][*CEN LEU2 SUP35$_{SC}$*]	This study	GT81(31)
GT1085	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ leu2-3,112 trp1-289 ura3 kar1 cyh^R sup35Δ::HIS3* [*rho$^-$*] [*psi$^-$*] [*PIN$^+$*] [*CEN URA3 SUP35N$_{SC}$MC$_{SC}$*]	This study	1B-D910
GT1086	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ leu2-3,112 trp1-289 ura3 kar1 cyh^R sup35Δ::HIS3* [*rho$^-$*] [*psi$^-$*] [*PIN$^+$*] [*CEN URA3 SUP35N$_{SP}$MC$_{SC}$*]		
GT1087	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ leu2-3,112 trp1-289 ura3 kar1 cyh^R sup35Δ::HIS3* [*rho$^-$*] [*psi$^-$*] [*PIN$^+$*] [*CEN URA3 SUP35N$_{SB}$MC$_{SC}$*]		
GT1088	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ leu2-3,112 trp1-289 ura3 kar1 cyh^R sup35Δ::HIS3* [*rho$^-$*] [*psi$^-$*] [*PIN$^+$*] [*CEN URA3 SUP35(NI)$_{SC}$(NII,III)$_{SP}$(MC)$_{SC}$*]		
GT1089	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ leu2-3,112 trp1-289 ura3 kar1 cyh^R sup35Δ::HIS3* [*rho$^-$*] [*psi$^-$*] [*PIN$^+$*] [*CEN URA3 SUP35(NI)$_{SP}$(NII,III)$_{SB}$(MC)$_{SC}$*]		
GT1090	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ leu2-3,112 trp1-289 ura3 kar1 cyh^R sup35Δ::HIS3* [*rho$^-$*] [*psi$^-$*] [*PIN$^+$*] [*CEN URA3 SUP35(NI)$_{SB}$(NII,III,MC)$_{SC}$*]		
GT1091	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ leu2-3,112 trp1-289 ura3 kar1 cyh^R sup35Δ::HIS3* [*rho$^-$*] [*psi$^-$*] [*PIN$^+$*] [*CEN URA3 SUP35(NI)$_{SB}$(NII,III)$_{SP}$(MC)$_{SC}$*]		

Table A.2 continued

GT1092	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ leu2-3,112 trp1-289 ura3 kar1 cyh^{R} sup35Δ::HIS3* [*rho^{-}*] [*psi^{-}*] [*PIN^{+}*] [*CEN URA3 SUP35(NI)$_{SP}$(NII,III,MC)$_{SC}$*]	This study	1B-D910
GT1093	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ leu2-3,112 trp1-289 ura3 kar1 cyh^{R} sup35Δ::HIS3* [*rho^{-}*] [*psi^{-}*] [*PIN^{+}*] [*CEN URA3 SUP35(NI)$_{SC}$(NII-III)$_{SB}$(MC)$_{SC}$*]		
GT1094	*S. cerevisiae MATα ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3* [*psi^{-}*][*PIN^{+}*][*CEN URA3 SUP35(NI)$_{SC}$(NII,III)$_{SP}$(MC)$_{SC}$*]	This study	GT81(31)
GT1095	*S. cerevisiae MATα ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3* [*psi^{-}*][*PIN^{+}*][*CEN URA3 SUP35(NI)$_{SP}$(NII,III)$_{SB}$(MC)$_{SC}$*]		
GT1096	*S. cerevisiae MATα ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3* [*psi^{-}*][*PIN^{+}*][*CEN URA3 SUP35(NI)$_{SB}$(NII,III,MC)$_{SC}$*]		
GT1097	*S. cerevisiae MATα ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3* [*psi^{-}*][*PIN^{+}*][*CEN URA3 SUP35(NI)$_{SB}$(NII,III)$_{SP}$(MC)$_{SC}$*]		
GT1098	*S. cerevisiae MATα ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3* [*psi^{-}*][*PIN^{+}*][*CEN URA3 SUP35(NI)$_{SP}$(NII,III,MC)$_{SC}$*]		
GT1099	*S. cerevisiae MATα ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3* [*psi^{-}*][*PIN^{+}*][*CEN URA3 SUP35(NI)$_{SC}$(NII,III)$_{SB}$(MC)$_{SC}$*]		
GT1103	*S. cerevisiae MATα ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3* weak [*PSI^{+}*][*CEN URA3 SUP35$_{SB}$*]	(1)	
GT1104	*S. cerevisiae MATα ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3* weak [*PSI^{+}*][*CEN URA3 SUP35$_{SP}$*]		
GT1114	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ leu2-3,112 trp1-289 ura3 kar1 cyh^{R} sup35Δ::HIS3* [*rho^{-}*] [*psi^{-}*] [*PIN^{+}*] [*CEN URA3 SUP35(NI)$_{SC}$(NII+1,III)$_{SB}$(MC)$_{SC}$ *]	This study	1B-D910
GT1115	*S. cerevisiae MATα ade1-14 $_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3* [*psi^{-}*][*PIN^{+}*] [*CEN URA3 SUP35(NI)$_{SC}$(NII+1,III)$_{SB}$(MC)$_{SC}$ *]	This study	GT81(31)
GT1148	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3* weak [*PSI^{+}*][*PIN^{+}*][*CEN URA3 SUP$_{SP}$*]		

Table A.2 continued

GT1155	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3 [PSI$^+$][CEN URA3 SUP35$_{SC}$]*	This study	GT81(31)
GT1156	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ leu2-3,112 trp1-289 ura3 kar1 cyh^R sup35Δ::HIS3 [RHO$^+$] [PSI$^+$] [PIN$^+$] [CEN URA3 SUP35$_{SC}$]*	This study	1B-D910
GT1157	*S. cerevisiae MATa ade1-14$_{SC}$ his3Δ leu2-3,112 trp1-289 ura3 kar1 cyh^R sup35Δ::HIS3 [RHO$^+$] [psi$^-$] [pin$^-$] [CEN URA3 SUP35$_{SC}$]*		
GT1182	*S. cerevisiae MATα ade1-14 $_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3 [psi$^-$][PIN$^+$] [CEN URA3 SUP35N(N12)$_{SC}$MC$_{SC}$]*	This study	GT81(31)
GT1192	*S. cerevisiae MATα ade1-14 $_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3 [psi$^-$][PIN$^+$] [CEN URA3 SUP35(NI,II)$_{SC}$(NIII)$_{SB}$(MC)$_{SC}$]*		
GT1208	*S. cerevisiae MATα ade1-14 $_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3 [PSI$^+$][PIN$^+$] [CEN URA3 SUP35N(N12)$_{SC}$MC$_{SC}$]*		
GT1209	*S. cerevisiae MATα ade1-14 $_{SC}$ his3Δ (or 11,15) lys2 leu2-3,112 trp1 ura3-52 sup35Δ::HIS3 [PSI$^+$][PIN$^+$] [CEN URA3 SUP35N(N12)$_{SC}$MC$_{SC}$]*		

Table A.3 *S. paradoxus* strains used and constructed in this work

Strain	Genotype/description	Reference/ Source
GT749-1B	*S. paradoxus MATα/MATa lys2/lys2 ura3-P2/ura3-P2*	G. Newnam
GT983	*S. paradoxus MATα/MATa lys2/lys2 ura3-P2/ura3-P2 HO/hoΔ::KANMX6*	This study
GT983-2A	*S. paradoxus MATa lys2 ura3-P2 hoΔ::KANMX6*	
GT992	*S. paradoxus MATa ade1Δ::URA3$_{SC}$ lys2 ura3-P2 hoΔ::KANMX6*	
GT1037	*S. paradoxus MATa ade1Δ::ura3$_{SC}$ lys2 ura3-P2 hoΔ::KANMX6*	
GT1116	*S. paradoxus MATa ade1Δ::ADE1$_{SC}$ lys2 ura3-P2 hoΔ::KANMX6*	
GT1142	*S. paradoxus MATa ade1Δ::ade1-14M$_{SC}$ lys2 ura3-P2 hoΔ::KANMX6*	
GT1174	*S. paradoxus MATa ade1Δ::ade1-14M$_{SC}$ lys2 ura3-P2 hoΔ::KANMX6 [PSI$^+$]*	

Table A.3 continued

GT1175	*S. paradoxus MATa ade1Δ::ade1-14M$_{SC}$ lys2 ura3-P2 hoΔ::KANMX6* [*PSI^{+}*]	This study
GT1188	*S. paradoxus MATa ade1Δ::ade1-14$_{SC}$ lys2 ura3-P2 hoΔ::KANMX6*	
OT23 (SP7-1D)	*S. paradoxus*	G. Naumov

Table A.4 *S. bayanus* strains used and constructed in this work

Strain	Genotype/description	Reference/ Source
GT986	*S. bayanus MATa ade1Δ::URA3$_{SC}$ ura3-1, hoΔ::KANMX4*	This study
GT991	*S. bayanus MATa ade1Δ::ADE1$_{SC}$ ura3-1, hoΔ::KANMX4*	
GT1020	*S. bayanus MATα, ura3-1 lys2 hoΔ::KANMX4*	
GT1028	*S. bayanus MATa ade1Δ::ade1-14$_{SC}$ ura3-1 hoΔ::KANMX4*	
GT1041	*S. bayanus MATα/MATa ADE1/ade1Δ::ade1-14$_{SC}$ LYS2/lys2 ura3-1/ura3-1 hoΔ::KANMX4/ hoΔ::KANMX4*	
GT1041-7A	*S. bayanus MATa ade1Δ::ade1-14$_{SC}$ lys2 ura3-1 hoΔ::KANMX4*	
GT1122	*S. bayanus MATα/MATa ADE1/ade1Δ::ade1-14$_{SC}$ LYS2/lys2 ura3-1/ura3-1 hoΔ::KANMX4/ hoΔ::KANMX4 SUP35/sup35Δ::natNT2*	
GT1122-4B	*S. bayanus MATα ade1Δ::ade1-14$_{SC}$ lys2 ura3-1 hoΔ::KANMX4 sup35Δ::natNT2* [*hphNT1 SUP35$_{SP}$*]	
GT1131	*S. bayanus MATα ade1Δ::ade1-14$_{SC}$ lys2 ura3-1 hoΔ::KANMX4 sup35Δ::natNT2* [*LYS2 SUP35$_{SP}$*]	
GT1132	*S. bayanus MATα ade1Δ::ade1-14$_{SC}$ lys2 ura3-1 hoΔ::KANMX4 sup35Δ::natNT2* [*LYS2 SUP35$_{SB}$*]	
GT1133	*S. bayanus MATα ade1Δ::ade1-14$_{SC}$ lys2 ura3-1 hoΔ::KANMX4 sup35Δ::natNT2* [*LYS2 SUP35$_{SC}$*]	
GT1144	*S. bayanus MATα ade1Δ::ade1-14$_{SC}$ lys2 ura3-1 hoΔ::KANMX4 sup35Δ::natNT2* [*hphNT1 SUP35$_{SC}$*]	
GT1150	*S. bayanus MATα ade1Δ::ade1-14$_{SC}$ lys2 ura3-1 hoΔ::KANMX4 sup35Δ::natNT2* [*rho^{-}*] [*hphNT1 SUP35$_{SB}$*]	
GT1158	*S. bayanus MATα ade1Δ::ade1-14$_{SC}$ lys2 ura3-1 hoΔ::KANMX4 sup35Δ::natNT2* [*rho^{-}*] [*hphNT1 SUP35$_{SP}$*]	
GT1159	*S. bayanus MATα ade1Δ::ade1-14$_{SC}$ lys2 ura3-1 hoΔ::KANMX4 sup35Δ::natNT2* [*rho^{-}*] [*hphNT1 SUP35$_{SC}$*]	
OT227 (FM361)	*S. bayanus*	M. Johnston
OT294 (Su1A)	*S. bayanus MATa, ura3-1, hoΔ::KANMX4*	(44)
OT295 (Su1B)	*S. bayanus MATα, ura3-1, hoΔ::KANMX4*	

APPENDIX B.

Plasmids used and constructed in this study

Table B.1 Plasmids used in this study

Plasmid name	Lab collection number	Yeast marker	Promoter	SUP35	Reference/ source
pRS316Gal	3	*URA3*	P_{GAL}	N/A	(52)
pBSKII(+)	53	*N/A*	*N/A*	*N/A*	Stratagene
pFL39	123	*TRP1*	N/A	N/A	(54)
pBluescript-URA3 I	132	*URA3*	P_{URA3}	*N/A*	J. Kumar
pYCH-U2	186	*URA3*	P_{SUP35}	$SUP35_{SC}$	(19)
pRS315	189	*LEU2*	*N/A*	*N/A*	(75)
p315Sp-SUP35HA3	288	*LEU2*	P_{SUP35}	$SUP35_{SC}HA$	(51)
p316Sp-SUP35	289	*URA3*	P_{SUP35}	$SUP35_{SC}$	
pET20b	290	N/A	P_{T7}	N/A	Novagen
pmCUPsGFP	294	*URA3*	P_{CUP1}	N/A	(53)
pmCUPNMsGFP	295	*URA3*	P_{CUP1}	$SUP35NM_{SC}$-GFP	
pET20b-Sup35NM	333	N/A	P_{T7}	$SUP35NM_{SC}$-$(His)_6$	(45)
pASB2	403	*LEU2*	P_{SUP35}	$SUP35_{SC}$	(43)
pFL39GAL-SUP35N	465	*TRP1*	P_{GAL}	$SUP35N_{SC}$	(43)
pYCL-CUP-SUP35NMSc	542	*LEU2*	P_{CUP1}	$SUP35NM_{SC}$	E. Lewitin
pYCL-CUP-NMScHPR6.6	545	*LEU2*	P_{CUP1}	$SUP35NM_{SC}$-*HPR6.6*	
pRS315-SUP35	596	*LEU2*	P_{SUP35}	$SUP35_{SC}$	S. Inge-Vechtomov, unpublished
pmCUP1MCSC	623	*URA3*	P_{CUP1}	$SUP35MC_{SC}$	(1)
pFA6a-KanMX6	658	N/A	P_{TEF}	*N/A*	(71)
CEN-GAL-Sup35-RFP	740	*URA3*	P_{GAL}	$SUP35_{SC}$-RFP	(50)
pRS317	917	*LYS2*	*N/A*	*N/A*	K. Lobachev
pRS303N	989	*natNT2*	*N/A*	*N/A*	(73)
pRS41H	996	*hphNT1*	*N/A*	*N/A*	

Table B.2 Plasmids constructed in this study

Plasmid name	Lab collection number	Yeast marker	Promoter	*SUP35*	Reference/ source
p316-PS-SUP35SP	752	*URA3*	P_{SUP35}	$SUP35_{SP}$	(1)
pRS316Gal-SUP35SP	775	*URA3*	P_{GAL}	$SUP35_{SP}$	This study
pRS316Gal-SUP35SB	776	*URA3*	P_{GAL}	$SUP35_{SB}$	(1)
p315-PS-SUP35SP	777	*LEU2*	P_{SUP35}	$SUP35_{SP}$	
pmCUP-SUP35SP	782	*URA3*	P_{CUP1}	$SUP35_{SP}$	
pFL39GAL-SUP35SB	808	*TRP1*	P_{GAL}	$SUP35_{SB}$	This study
p316-PS-SUP35SB	811	*URA3*	P_{SUP35}	$SUP35_{SB}$	(1)
p315-PS-SUP35SB	813	*LEU2*	P_{SUP35}	$SUP35_{SB}$	
pmCUP-NMSPsGFP	836	*URA3*	P_{CUP1}	$SUP35NM_{SP}$-GFP	
pmCUP-NMSBsGFP	837	*URA3*	P_{CUP1}	$SUP35NM_{SB}$-GFP	
pFL39-CUP-NMSPsGFP	857	*TRP1*	P_{CUP1}	$SUP35NM_{SP}$-GFP	
pFL39-CUP-NMSBsGFP	858	*TRP1*	P_{CUP1}	$SUP35NM_{SB}$-GFP	
pFL39-CUP-NMSCsGFP	868	*TRP1*	P_{CUP1}	$SUP35NM_{SC}$-GFP	
pmCUP-NMPMsGFP	900	*URA3*	P_{CUP1}	$SUP35NM_{PM}$-GFP	
pFL39-CUP-NMPMsGFP	909	*TRP1*	P_{CUP1}	$SUP35NM_{PM}$-GFP	
pET20b-Sup35NMSP	910	N/A	P_{T7}	$SUP35NM_{SP}$-$(His)_6$	
pET20b-Sup35NMSB	911	N/A	P_{T7}	$SUP35NM_{SB}$-$(His)_6$	
pmCUP-NMSP-HPR6.6	912	*URA3*	P_{CUP1}	*$SUP35NM_{SP}$-HPR6.6*	This study
pmCUP-NMSB-HPR6.6	913	*URA3*	P_{CUP1}	*$SUP35NM_{SB}$-HPR6.6*	
pRS316-CUP-NMSC-HPR6.6	922	*URA3*	P_{CUP1}	*$SUP35NM_{SC}$-HPR6.6*	
pBSKII(+)-ADE1SC	960	N/A	P_{ADE1}	*N/A*	This study
pBSKII(+)-ade1-14SC	961	N/A	P_{ADE1}	*N/A*	

Table B.2 continued

pRS317-ADE1SC	963	*LYS2*	P_{ADE1}	*N/A*	This study
pRS317-ade1-14SC	964	*LYS2*	P_{ADE1}	*N/A*	
p316-PS-SUP35NSC-MCSC	966	*URA3*	P_{SUP35}	$SUP35N_{SC}MC_{SC}$	(1)
p316-PS-SUP35NSP-MCSC	967	*URA3*	P_{SUP35}	$SUP35N_{SP}MC_{SC}$	
p316-PS-SUP35NSB-MCSC	968	*URA3*	P_{SUP35}	$SUP35N_{SB}MC_{SC}$	
p316-PS-SUP35(NI)SC(NII,III)SP(MC)SC	977	*URA3*	P_{SUP35}	$SUP35(NI)_{SC}(NII,III)_{SP}(MC)_{SC}$	This study
p316-PS-SUP35(NI)SP(NII,III)SB(MC)SC	979	*URA3*	P_{SUP35}	$SUP35(NI)_{SP}(NII,III)_{SB}(MC)_{SC}$	
p316-PS-SUP35(NI)SB(NII,III,MC)SC	980	*URA3*	P_{SUP35}	$SUP35(NI)_{SB}(NII,III,MC)_{SC}$	
p316-PS-SUP35(NI)SB(NII,III)SP(MC)SC	981	*URA3*	P_{SUP35}	$SUP35(NI)_{SB}(NII,III)_{SP}(MC)_{SC}$	
p316-PS-SUP35(NI)SP(NII,III,MC)SC	983	*URA3*	P_{SUP35}	$SUP35(NI)_{SP}(NII,III,MC)_{SC}$	
p316-PS-SUP35(NI)SC(NII,III)SB(MC)SC	984	*URA3*	P_{SUP35}	$SUP35(NI)_{SC}(NII,III)_{SB}(MC)_{SC}$	
pFL39GAL-SUP35SP	1005	*TRP1*	P_{GAL}	$SUP35_{SP}$	
pRS41H-SUP35SP	1006	*hphNT1*	P_{SUP35}	$SUP35_{SP}$	
pRS41H-SUP35SC	1007	*hphNT1*	P_{SUP35}	$SUP35_{SC}$	
pRS316GAL-SUP35(NI)SP(NII,III)SB(MC)SC	1008	*URA3*	P_{GAL}	$SUP35(NI)_{SP}(NII,III)_{SB}(MC)_{SC}$	
pRS316GAL-SUP35(NI)SB(NII,III)SP(MC)SC	1009	*URA3*	P_{GAL}	$SUP35(NI)_{SB}(NII,III)_{SP}(MC)_{SC}$	
pRS315GAL-SUP35(NI)SP(NII,III)SB(MC)SC	1010	*LEU2*	P_{GAL}	$SUP35(NI)_{SP}(NII,III)_{SB}(MC)_{SC}$	
pRS315GAL-SUP35(NI)SB(NII,III)SP(MC)SC	1011	*LEU2*	P_{GAL}	$SUP35(NI)_{SB}(NII,III)_{SP}(MC)_{SC}$	

Table B.2 continued

p316-PS-SUP35(NI)SC(NII+1,III)SB(MC)SC	1013	*URA3*	P_{SUP35}	$SUP35(NI)_{SC}(NII+1,III)_{SB}(MC)_{SC}$	This study
pRS317-PS-SUP35SP	1015	*LYS2*	P_{SUP35}	$SUP35_{SP}$	
pRS317-PS-SUP35SB	1016	*LYS2*	P_{SUP35}	$SUP35_{SB}$	
pRS317-PS-SUP35SC	1017	*LYS2*	P_{SUP35}	$SUP35_{SC}$	
p316-PS-SUP35(NI,II)SC(NIII)SB(MC)SC	1019	*URA3*	P_{SUP35}	$SUP35(NI,II)_{SC}(NIII)_{SB}(MC)_{SC}$	
p316-PS-SUP35(NI(S12N))SP(NII,III,MC)SC	1026	*URA3*	P_{SUP35}	$SUP35(NI(S12N))_{SP}(NII,III,MC)_{SC}$	
pRS41H-SUP35SB	1034	*hphNT1*	P_{SUP35}	$SUP35_{SB}$	
P316-PS-SUP35(N(N12S))SC(MC)SC	1050	*URA3*	P_{SUP35}	$SUP35(N(N12S)_{SC}(MC)_{SC}$	
pRS315GAL-SUP35(NI)SC(NII,III)SB(MC)SC	1051	*LEU2*	P_{GAL}	$SUP35(NI)_{SC}(NII,III)_{SB}(MC)_{SC}$	
pRS315GAL-SUP35(NI)SC(NII,III)SP(MC)SC	1052	*LEU2*	P_{GAL}	$SUP35(NI)_{SC}(NII,III)_{SP}(MC)_{SC}$	
pRS315GAL-SUP35(NI)SB(NII,III,MC)SC	1053	*LEU2*	P_{GAL}	$SUP35(NI)_{SB}(NII,III,MC)_{SC}$	
pRS315GAL-SUP35(NI)SP(NII,III,MC)SC	1054	*LEU2*	P_{GAL}	$SUP35(NI)_{SP}(NII,III,MC)_{SC}$	

APPENDIX C.

Primers used in this study

Table C. Primers used in this study

Name	Lab collection number	Sequence	Used for amplifying
SUP35-C-NDEI	59	5’ - CGG CCA TAT GTC GGA TTC AAA CCA AGG C - 3'	NM domain beginning with *S. cerevisiae* sequence (Forward)
SUP35-C-XHOI	60	5’ - ACA CTC GAG ATC GTT AAC AAC TTC GTC ATC - 3'	NM domain (Reverse)
SUP35-PAR-F	219	5’-TATCGGATCCCTAGCAACAATGTCGGATTCA-3’	$SUP35_{SP}$ ORF (Forward)
SUP35-PAR-R	220	5’-ATATGAGCTCCAGCTTTATTAAACCGGGTTT-3’	$SUP35_{SP}$ ORF (Reverse)
SUP35-BAY-F	221	5’-ACCTGGATCCTAGCAATCATGTCTGACCCA-3’	$SUP35_{SB}$ ORF (Forward)
SUP35-BAY-R	222	5’-GTAAGAGCTCGAAAGGGCTATGACGAAAAC-3’	$SUP35_{SB}$ ORF (Reverse)
Sup35Spar NM-REVGN	309	5’-CCCCCCGCGGGTCATTAACAACTTCGTCA-3’	$SUP35NM_{SP}$ (Reverse)
Sup35Sbay NM-REVGN	310	5’-CTCCCCGCGGATCATTAACAACTTCGTCGT-3’	$SUP35NM_{SB}$ (Reverse)
SUP35NMPmGFPF	367	5'-CACCGGATCCATGTCTCAAGATCAACAGCA-3	$SUP35NM_{PM}$ (Forward)
SUP35NMPmGFPR	368	5'-AAGCCCGCGGTTCGGATTCACTGACTGGCT-3'	$SUP35NM_{PM}$ (Reverse)
HOSparCheF	369	5’ - ACTTCTATTA CAACCATTAC-3’	$KanMX6/HO_{SP}$ (Forward)
HOSparCheR	370	5’ - ATCTTTTTTA TCCAAAATAT-3’	$KanMX6/HO_{SP}$ (Reverse)
HOSparDelF	371	5’- CTCTAAATCC ACACCCTTAT AAGCAGCAAT CAATTTCATC TAACTTCAAC CGGATCCCCG GGTTAATTAA-3’	*KanMX6* (Forward)

Table C. continued

HOSparDelR	372	5’ - TAAAATTTAC ATTTATCACA TACAACTTTT TTTTAACTAA TGTACACATT GAATTCGAGC TCGTTTAAAC-3’	*KanMX6* (Reverse)
Sup35NMSpar-F-NdeI(His)	374	5’-AGCACATATGTCGGATTCAAACC AAGGTAA-3’	*SUP35NM*$_{SP}$ (Forward)
Sup35NMSpar-R-XhoI(His)	375	5’-CCCCCTCGAGGTCATTAACAACTT CGTCAT-3’	*SUP35NM*$_{SP}$ (Reverse)
Sup35NMSbay-F-NdeI(His)	376	5’-AGCACATATGTCTGACCCAAATC AAGGTAA-3’	*SUP35NM*$_{SB}$ (Forward)
Sup35NMSbay-R-XhoI(His)	377	5’-CTCCCTCGAGATCATTAACAACTT CGTCGT-3’	*SUP35NM*$_{SB}$ (Reverse)
ADE1Sbayche-F	378	5’ – ATA CAA AGA GAG AAG CAA GA-3’	*URA3*$_{SC}$ */ADE1*$_{SB}$ (Forward)
ADE1Sbayche-R	379	5’ – GTA ACA AAT AGA AAG AAC GC-3’	*URA3*$_{SC}$ */ADE1*$_{SB}$ (Reverse)
ADE1-14Scer-F	382	5’-CTG GGC CAA CCG CAT CGG AAG CAC TGC TTA GAG GGA TAT CAT ACA AAG AGA GAA GCA AGA ATG TCA ATT ACG AAG ACT GA-3’	*ADE1*$_{SC}$ */ade1-14*$_{SC}$ (Forward)
ADE1-14Scer-R	383	5’ –TAC GTA TGT ATA TAT TTA GTG CGA GAT TCA CTG ATG ACC TGT AAC AAA TAG AAA GAA CGC TTA GTG AGA CCA TTT AGA CC-3’	*ADE1*$_{SC}$ */ade1-14*$_{SC}$ (Reverse)
ADE1SBDEL-F#2	386	5’ – GCA CTG CTT AGA GGG ATA TCA TAC AAA GAG AGA AGC AAG AGT AAT ACG ACT CAC TAT AGG GC-3’	*URA3*$_{SC}$ (Forward)
ADE1SBDEL-R#2	387	5’ –GCG AGA TTC ACT GAT GAC CTG TAA CAA ATA GAA AGA ACG CTC GAG GTC GAC GGT ATC-3’	*URA3*$_{SC}$ (Reverse)
HPR-Forward	390	5’- CGATCCGCGG ATGGCCATGG CTGCCGAGGA - 3’	*HPR6.6* (Forward)
HPR-Reverse	391	5’- TGCAGAGCTC CTGTTACAAA TGATTTTGCA - 3’	*HPR6.6* (Reverse)

Table C. continued

ade1SparChe-F	392	5’ - CATACGAAAA AGTAATAACA - 3’	*URA3*$_{SC}$ */ADE1*$_{SP}$ (Forward)
ade1SparChe-R	393	5’- GTAGCATATG TAAAAACACT - 3’	*URA3*$_{SC}$ */ADE1*$_{SP}$ (Reverse)
ADE1SPARDEL-F	394	5’ CATTACTTAT AAAGAATACA CATACGAAAA AGTAATAACA GTAATACGAC TCACTATAGGGC 3’	*URA3*$_{SC}$ (Forward)
ADE1SPARDEL-R	395	5’ - GCGAAGTACA CTGGCGACTT GTAGCATATG TAAAAACACT TCGAGGTCGA CGGTATC - 3’	*URA3*$_{SC}$ (Reverse)
ADE1-14SPAR-F	396	5’ - AGAATCAATT GAATCATAAG CATTACTTAT AAAGAATACA CATACGAAAA AGTAATAACA ATGTCAATTA CGAAGACTGA - 3’	*ADE1*$_{SC}$ */ade1-14*$_{SC}$ (Forward)
ADE1-14SPAR-R	397	5’ - ATGTATGATT CATATTTAGT GCGAAGTACA CTGGCGACTT GTAGCATATG TAAAAACACT TTAGTGAGAC CATTTAGACC - 3’	*ADE1*$_{SC}$ */ade1-14*$_{SC}$ (Reverse)
ADE1-F	448	5’ – TAC TCT GCA GCT TAC CAA GCA GAG AAT GTT ATC T-3’	*ADE1*$_{SC}$ */ade1-14*$_{SC}$ with 500bp upstream of ORF (Forward)
ADE1-R	449	5’ – GCA GGA GCT CAG CGA GCC AGG GAA GTA ATT-3’	*ADE1*$_{SC}$ */ade1-14*$_{SC}$ with 200bp downstram of ORF (Reverse)
Sup35NSB-Sup35MCSC-R	467	5’-TCAAAGATCTTCCTTGAGATTGTG GTTGAA-3’	*SUP35N*$_{SB}$ (Reverse)
Sup35NSC-Sup35MCSC-R	468	5’-TCAAAGATCTACCTTGAGACTGTG GTTGGA-3’	*SUP35N*$_{SC}$ and *SUP35N*$_{SP}$ (Reverse)

Table C. continued

SB-Insertion-R	508	5’ - TACCA CGGCC ACCTT GTGGG TTGAA TTGCT GTTGG TAACC gcctt gagga ttgta ctgtt gatag ccGCC TTGAG CGTTG TATTG TTGTT GGTAA CCTGC TTCCG GG - 3’	I,II module ending with *S. bayanus* sequence with additional repeated unit from *S. cerevisiae* (Reverse)
SP-S12N-R	509	5’ - CACGG CCACC TTGTG GATTGA - 3’	I,II module ending with *S. cerevisiae* sequence (Reverse)
ADE1-14SPAR+140-F	515	5’ – TATATATATA TGTACATTCT CACCTGGATT CTTTGGGGGT AAAACGGTTG AGTGTTGTGC TTTTGTAGTT GGTACTGTTA AGAATCAATT GAATCATAAG - 3’	$ADE1_{SC}$ /$ade1\text{-}14_{SC}$ (Forward)
ADE1-14SPAR+140-R	516	5’ – CGCCAAACCT G CATACCACT GGCAAACAAG ATATCGATAA GACTTGCTTT GAGAACATTT ATACATTAAT ACATATGGGT ATGTATGATT CATATTTAGT - 3’	$ADE1_{SC}$ /$ade1\text{-}14_{SC}$ (Reverse)
sup35SB::natNT2-check-F	533	5’- ATACCTGCCC ACTAGCAATC - 3’	*natNT2* /$SUP35_{SB}$ (Forward)
sup35SB::natNT2-check-R	534	5’- GTTTAATTCT TGCGAAAAAA - 3’	*natNT2* /$SUP35_{SB}$ (Reverse)
sup35SB::natNT2-F	535	5’ - GTTTACTAGC AACAGTACCT ATACCTGCCC ACTAGCAATC GGATCCCCGG GTTAATTAAG - 3’	*natNT2* (Forward)
sup35SB::natNT2-R	536	5’ – TGGGGTTGTT TTTTTTTTTC GTTTAATTCT TGCGAAAAAA GAGCTCGATT ACAACAGGTG - 3’	*natNT2* (Reverse)
SP-S12N-F#2	545	5’ - TATC GGATCC CTAGC AACA ATGTC GGATT CAAAC CAAGG TAACA ATCAG CAAA**A** CTACC AGCAA TACGG CCAAA ACTCT - 3’	N domain beginning with *S. paradoxus* sequence with S12N substitution (Forward)

Table C. continued

NSC-R-BglII-SacI	546	5’ - AGTC GAGCTC AGATCT ACCTT GAGAC TGTGG TTGGA - 3’	N domain ending with *S. cerevisiae* sequence (Reverse)
NSC-MCSC(N12S)	551	5’ - AGCAGGATCCCTAGCAACA ATGTCGGATT CAAACCAAGG CAACAATCAG CAAA**G**CTACCAGCAATACAGCCA GAA - 3’	N domain beginning with *S. cerevisiae* sequence with N12S substitution (Forward)

Restriction sites are underlined. Insertion sequence of the additional repetitive unit from *S. cerevisiae* is shown in lower case. Nucleotides used to create S12N and N12S mutations are shown in bold.

APPENDIX D.

Insertion of one *S. cerevisiae* repetitive unit to the ORs region of *S. bayanus*

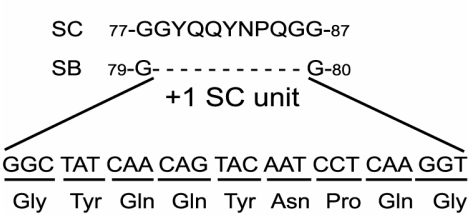


Figure D. Insertion of one *S. cerevisiae* repetitive unit to the ORs region of *S. bayanus*

SC and SB refer to *S. cerevisiae* and *S. bayanus*, respectively. Numbers correspond to amino acid positions. Missing residues are indicated by dashes. Each codon is underlined. "+1 SC unit" refers to one additional *S. cerevisiae* repetitive unit.

APPENDIX E.

Mitotic stability of the [*PSI*$^{+}$] prions generated by chimeric Sup35 proteins

Table E. Mitotic stability of the [*PSI*$^+$] prions generated by chimeric Sup35 proteins

SUP35N			Prion isolate	Number	Stability		
I	***II***	***III***			**[*PSI*$^+$]**	**[*psi*$^-$](%)**	**Total**
SC	SP	SP	Strong	1	>100	0	>100
				2	>100	0	>100
				3	>100	0	>100
				4	>100	0	>100
				5	>100	0	>100
				6	>100	0	>100
				7	120	0	120
				8	126	0	126
				9	123	0	123
				10	157	0	157
				11	151	0	151
				12	142	0	142
				13	120	0	120
			Weak	14	152	0	152
				15	174	0	174
				16	127	0	127
				17	168	0	168
SB	SC	SC	Strong	1	>100	0	>100
				2	>100	0	>100
				3	>100	0	>100
				4	>100	0	>100
				5	>100	0	>100
				6	>100	0	>100
SP	SC	SC	Strong	1	41	1(2.5%)	42
				2	98	0	98
				3	>100	0	>100
				4	95	1(1%)	96
				5	180	0	180
				6	115	0	115
				7	190	11(5.5%)	201
				8	120	0	120
				9	130	0	130
				10	107	1(0.9%)	108
				11	211	0	211
				12	198	0	198
				13	130	2(1.5%)	132
				14	137	9(6.2%)	146
				15	166	0	166
			Weak	16	132	0	132
				17	110	0	110
				18	214	0	214

Table E. continued

SP	SB	SB	Strong	1	123	0	123
			Weak	2	54	115(68.0%)	169
				3	33	171(83.8%)	204
				4	35	183(83.9%)	218
				5	113	0	113
				6	127	0	127
				7	205	0	205
				8	118	0	118
				9	140	0	140
SC	SB	SB	Strong	1	>100	0	>100
				2	>100	0	>100
				3	115	0	115
				4	112	0	112
				5	136	0	136
			Weak	6	>100	0	>100
				7	116	0	116
				8	129	0	129
				9	137	0	137
SC	SC	SB	Strong	1	105	0	105
				2	145	0	145
				3	128	0	128
				4	128	0	128
				5	122	0	122
				6	140	0	140
				7	128	0	128
				8	114	0	114
				9	137	0	137
				10	131	0	131
				11	140	0	140
				12	118	0	118
				13	106	0	106
				14	130	0	130
				15	123	1(0.8%)	124
				16	124	0	124
				17	140	0	140
				18	108	0	108
				19	128	0	128
				20	110	0	110
				21	108	0	108
				22	110	0	110

Table E. continued

SC	SB+ 1SC unit	SB	Strong	1	72	0	72
				2	90	0	90
			Weak	3	82	13(13.7%)	95
				4	81	17(17.3%)	98
				5	41	54(56.8%)	95
				6	40	70(63.6%)	110
				7	86	20(18.9%)	106
				8	70	17(19.5%)	87
				9	80	15(15.8%)	95
				10	84	16(16.0%)	100
SB	SP	SP	Strong	1	111	0	111
				2	>200	0	>200
				3	>200	0	>200
				4	322	0	322
				5	>200	0	>200
				6	62	0	62
				7	173	0	173
				8	112	0	112
				9	>100	0	>100
				10	>200	0	>200
				11	>200	0	>200
				12	>100	0	>100
				13	157	1(0.6%)	158
				14	145	0	145
				15	123	0	123
				16	136	0	136
				17	161	0	161
				18	140	0	140
				19	131	0	131
				20	161	0	161
				21	121	0	121
				22	138	0	138

In each case, [*PSI*$^+$] culture was grown on –Ura-Trp-Ade(-Ura-Leu-Ade) medium for 14 days, streaked out on YPD medium for single colonies, and from YPD to –Ade, Ade$^+$ colonies were checked for presence of [*PSI*$^+$]. In all cases, Sup35 has a chimeric prion domain (*SUP35N*) with modules (I, II and III) from different origins (SC-*S. cerevisiae*, SP-*S. paradoxus* and SB-*S. bayanus*) as indicated fused to *SUP35MC* of *S. cerevisiae*. "+1 SC" unit indicates the *S. bayanus* region II with one extra repetitive unit of *S. cerevisiae* origin added. All [*PSI*$^+$] isolates listed in this table were turned into [*psi*$^-$] after 3 passages on YPD medium with 5 mM GuHCl. "Strong" and "weak" refer to the ability to grow on –Ade medium.

APPENDIX F.

Mitotic stability of the [*PSI*$^+$] prions produced by plasmid shuffle

Table F. Mitotic stability of the [*PSI*$^+$] prions produced by plasmid shuffle

SUP35N			Prion isolate	Number	Stability		
I	***II***	***III***			**[*PSI*$^+$]**	**[*psi*$^-$](%)**	**Total**
SC	SP	SP	Strong	1	>100	0	>100
				2	239	0	239
				3	>100	0	>100
				4	>100	0	>100
				5	>100	0	>100
			Weak	6	>100	0	>100
				7	>100	0	>100
				8	>100	0	>100
				9	>100	0	>100
				10	>100	0	>100
				11	>100	0	>100
				12	>100	0	>100
SP	SC	SC	Strong	1	>100	0	>100
				2	>100	0	>100
			Weak	3	>100	0	>100
SB	SC	SC	Strong	1	>100	0	>100
				2	258	0	258
				3	>100	0	>100
				4	>100	0	>100
				5	>100	0	>100
				6	>100	0	>100
				7	>100	0	>100
				8	>100	0	>100
				9	>100	0	>100
				10	>100	0	>100
				11	>100	0	>100
				12	>100	0	>100
				13	>100	0	>100
				14	>100	0	>100
				15	>100	0	>100
				16	>100	0	>100
				17	>100	0	>100
				18	231	0	231
SB	SP	SP	Strong	1	>100	0	>100
				2	>100	0	>100
				3	>100	0	>100
SC	SB +1SC unit	SB	Strong	1	244	25(9.3)	269
				2	>100	0	>100
			Weak	3	139	56(28.7)	195

Table F. continued

SC	SC	SB	Strong	1	>100	0	>100
				2	>100	0	>100
				3	>100	0	>100
				4	>100	0	>100
				5	>100	0	>100
				6	>100	0	>100
				7	>100	0	>100
				8	>100	0	>100
				9	>100	0	>100
				10	>100	0	>100
SP (S12N)	SC	SC	Strong	1	141	0	141
				2	116	1(0.8)	116
				3	88	0	88
				4	148	0	148
				5	84	0	84
				6	>100	0	>100
				7	154	0	154
				8	94	0	94
				9	>100	0	>100
				10	>100	0	>100
				11	47	0	47
				12	>100	0	>100
				13	>100	0	>100
				14	109	1(0.9)	110

Ade^+ colonies produced by shuffle from *S. cerevisiae SUP35* were checked for presence of [PSI^+]. In all cases, Sup35 has a chimeric prion domain (*SUP35N*) with modules (I, II and III) from different origins (SC-*S. cerevisiae*, SP-*S. paradoxus* and SB-*S. bayanus*) as indicated fused to *SUP35MC* of *S. cerevisiae*. "+1 SC" unit indicates the *S. bayanus* region II with one extra repetitive unit of *S. cerevisiae* origin added. "S12N", a single amino acid substitution was shown within its respective module. All [PSI^+] isolates listed in this table were obtained by shuffle from the strong [PSI^+] and turned into [psi^-] after 3 passages on YPD medium with 5 mM GuHCl. "Strong" and "weak" refer to the ability to grow on –Ade medium. Mosaic colonies (usually rare in strong and stable [PSI^+] isolates) were counted as [PSI^+].

APPENDIX G.

Sequences of the *ADE1* gene (A) and Ade1 protein (B) of *S. cerevisiae*

A

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   1 ATGTCAATTA CGAAGACTGA ACTGGACGGT ATATTGCCAT TGGTGGCCAG
  51 AGGTAAAGTT AGAGACATAT ATGAGGTAGA CGCTGGTACG TTGCTGTTTG
 101 TTGCTACGGA TCGTATCTCT GCATATGACG TTATTATGGA AAACAGCATT
 151 CCTGAAAAGG GGATCCTATT GACCAAACTG TCAGAGTTCT GGTTCAAGTT
 201 CCTGTCCAAC GATGTTCGTA ATCATTTGGT CGACATCGCC CCAGGTAAGA
 251 CTATTTTCGA TTATCTACCT GCAAAATTGA GCGAACCAAA GTACAAAACG
 301 CAACTAGAAG ACCGCTCTCT ATTGGTTCAC AAACATAAAC TAATTCCATT
 351 GGAAGTAATT GTCAGAGGCT ACATCACCGG ATCTGCTTGG AAAGAGTACG
 401 TAAAAACAGG TACTGTGCAT GGTTTGAAAC AACCTCAAGG ACTTAAAGAA
 451 TCTCAAGAGT TCCCAGAACC AATCTTCACC CCATCGACCA AGGCTGAACA
 501 AGGTGAACAT GACGAAAACA TCTCTCCTGC CCAGGCCGCT GAGCTGGTGG
 551 GTGAAGATTT GTCACGTAGA GTGGCAGAAC TGGCTGTAAA ACTGTACTCC
 601 AAGTGCAAAG ATTATGCTAA GGAGAAGGGC ATCATCATCG CAGACACTAA
 651 ATTCGAATTC GGTATTGACG AAAAGACCAA TGAAATTATT CTAGTGGACG
 701 AGGTGCTAAC GCCAGACTCC TCTAGATTCT GGAACGGTGC CTCTTATAAG
 751 GTAGGAGAAT CCCAAGATTC TTACGATAAG CAATTTTTAA GAGACTGGCT
 801 TACTGCTAAT AAGTTGAACG GTGTTAACGG CGTCAAAATG CCCCAAGACA
 851 TTGTCGACAG GACAAGGGCC AAATATATAG AGGCTTATGA AACATTGACA
 901 GGGTCTAAAT GGTCTCACTA A
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B

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   1 MSITKTELDG ILPLVARGKV RDIYEVDAGT LLFVATDRIS AYDVIMENSI
  51 PEKGILLTKL SEFWFKFLSN DVRNHLVDIA PGKTIFDYLP AKLSEPKYKT
 101 QLEDRSLLVH KHKLIPLEVI VRGYITGSAW KEYVKTGTVH GLKQPQGLKE
 151 SQEFPEPIFT PSTKAEQGEH DENISPAQAA ELVGEDLSRR VAELAVKLYS
 201 KCKDYAKEKG IIIADTKFEF GIDEKTNEII LVDEVLTPDS SRFWNGASYK
 251 VGESQDSYDK QFLRDWLTAN KLNGVNGVKM PQDIVDRTRA KYIEAYETLT
 301 GSKWSH
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Figure G. Sequences of the *ADE1* gene (A) and Ade1 protein (B) of *S. cerevisiae*
Numbers correspond to nucleotide (A) and amino acid (B) positions. Squares indicate a codon or two consequent codons. Substitutions are shown in bold. For both *ade1-14*$_{SC}$ and *ade1-14M*$_{SC}$, G732A leads to a pre-mature stop codon at W244. For *ade1-14M*$_{SC}$ only, G830A and G832A lead to G277N and V278I, respectively.

Total of 22 *S. paradoxus* potential candidate strains with *ade1-14*$_{SC}$ allele were verified by PCR, and 5 of them were capable of prion induction. 4 out of them were sequenced, producing the *ade1-14*$_{SC}$ without changes and *ade1-14M*$_{SC}$. The other 2 sequenced potential candidates lost the ability of prion induction, one of them had K184Q mutation, the other one had H306P and the stop codon mutated to 307K which caused lack of termination (readthrough).

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